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C07K 14/475, A61K 38/18, C12N 15/12, C12Q 1/68, // (A61K 38/18 38:16 38:19 38:20 38:22) (C12N 15/12 C12R 1:19 1:91)

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C3H HA3 HB7P HFZ H100 H106 H107 H108 H140 H317 H320 H321 H322 H324 H325 H339 H370 H380 H650 H656 H672
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U1S S1332 S2415

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(58) continued overleaf

(54) Thrombopoietin

(57) Isolated thrombopoietin (TPO), isolated DNA encoding TPO, and recombinant or synthetic methods of preparing and purifying TPO are disclosed. Various forms of TPO are shown to influence the replication. differentiation or maturation of blood cells, especially megakaryocytes and megakaryocyte progenitor cells. Accordingly, these compounds may be used for treatment of thrombocytopenia.

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1990.

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GB 2 285 446 A

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  1 tcttcctacccatctgctccccagagggctgcctgctgtgcacttgggtcctggagcccttctccacccggatagattcctcacccttggcccgcctttg

101 ccccaccctactctgcccagaagtgcaagagcctaagccgcctccatggccccaggaaggattcaggggagaggccccaaacagggagccacgccagcca

                   -20                     -10
                MetGluLeuThrGluLeuLeuLeuValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCysAsp
201 gacaccccggccagaATGGAGCTGACTGAATTGCTCCTCGTGGTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGTG

       10                  20                         30                  40
  LeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProThrProValLeuLeu
301 ACCTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGCCAGTGCCCAGAGGTTCACCCTTTGCCTACACCTGTCCTGCT

                  50                            60                           70
  ProAlaValAspPheSerLeuGlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyVal
401 GCCTGCTGTGGACTTTAGCTTGGGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTGACCCTTCTGCTGGAGGGAGTG

            80                            90                            100
  MetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeu
501 ATGGCAGCACGGGGACAACTGGGACCCACTTGCCTCTCATCCCTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTCC

       110                           120                           130                           140
   GlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIlePheLeuSerPheGlnHisLeuLeuArgGlyLysValArgPhe
601 TTGGAACCCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATCTTCCTGAGCTTCCAACACCTGCTCCGAGGAAAGGTGCGTTT

                  150                           160                           170
   LeuMetLeuValGlyGlySerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeuValLeuThrLeuAsnGluLeu
701 CCTGATGCTTGTAGGAGGGTCCACCCTCTGCGTCAGGCGGGCCCCACCCACCACAGCTGTCCCCAGCAGAACCTCTCTAGTCCTCACACTGAACGAGCTC

            180                           190                           200
   ProAsnArgThrSerGlyLeuLeuGluThrAsnPheThrAlaSerAlaArgThrThrGlySerGlyLeuLeuLysTrpGlnGlnGlyPheArgAlaLysIle
801 CCAAACAGGACTTCTGGATTGTTGGAGACAAACTTCACTGCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTCAGAGCCAAGA
```

FIG. 1A

```
     210                                      220                                    230                                      240
   ProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsnArgIleHisGluLeuLeuAsnGlyThrArgGlyLeuPhePro
901 TTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAACAGGATACACGAACTCTTGAATGGAACTCGTGGACTCTTTCC

                                          250                                    260                                   270
   GlyProSerArgArgThrLeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeuGlnProGlyTyrSerProSer
1001 TGGACCCTCACGCAGGACCCTAGGAGCCCCGGACATTTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCTCCAGCCTGGATATTCTCCTTCC

                         280                                     290                                  300
   ProThrHisProProThrGlyGlnTyrThrLeuPheProLeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuProAspProSerAla
1101 CCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCCTCTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGCTTCCTGACCCTTCTG

     310                                      320                                   330
   ProThrProThrProThrSerProLeuLeuAsnThrSerTyrThrHisSerGlnAsnLeuSerGlnGluGly
1201 CTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAATCTGTCTCAGGAAGGGTAAggttctcagacactgccgacatc

1301 agcattgtctcatgtacagctcccttccctgcagggcgcccctgggagacaactggacaagatttcctactttctcctgaaacccaaagccctggtaaaa

1401 gggatacacaggactgaaaagggaatcatttttcactgtacattataaaccttcagaagctatttttttaagctatcagcaatactcatcagagcagcta

1501 gctctttggtctattttctgcagaaatttgcaactcactgattctctacatgctctttttctgtgataactctgcaaaggcctgggctggcctggcagtt

1601 gaacagagggagagactaaccttgagtcagaaaacagagaaagggtaatttcctttgcttcaaattcaaggccttccaacgcccccatcccctttactat

1701 cattctcagtgggactctgatcccatattcttaacagatctttactcttgagaaatgaataagctttctctcagaaaaaaaaaaaaaaaaaaa
```

FIG. 1B

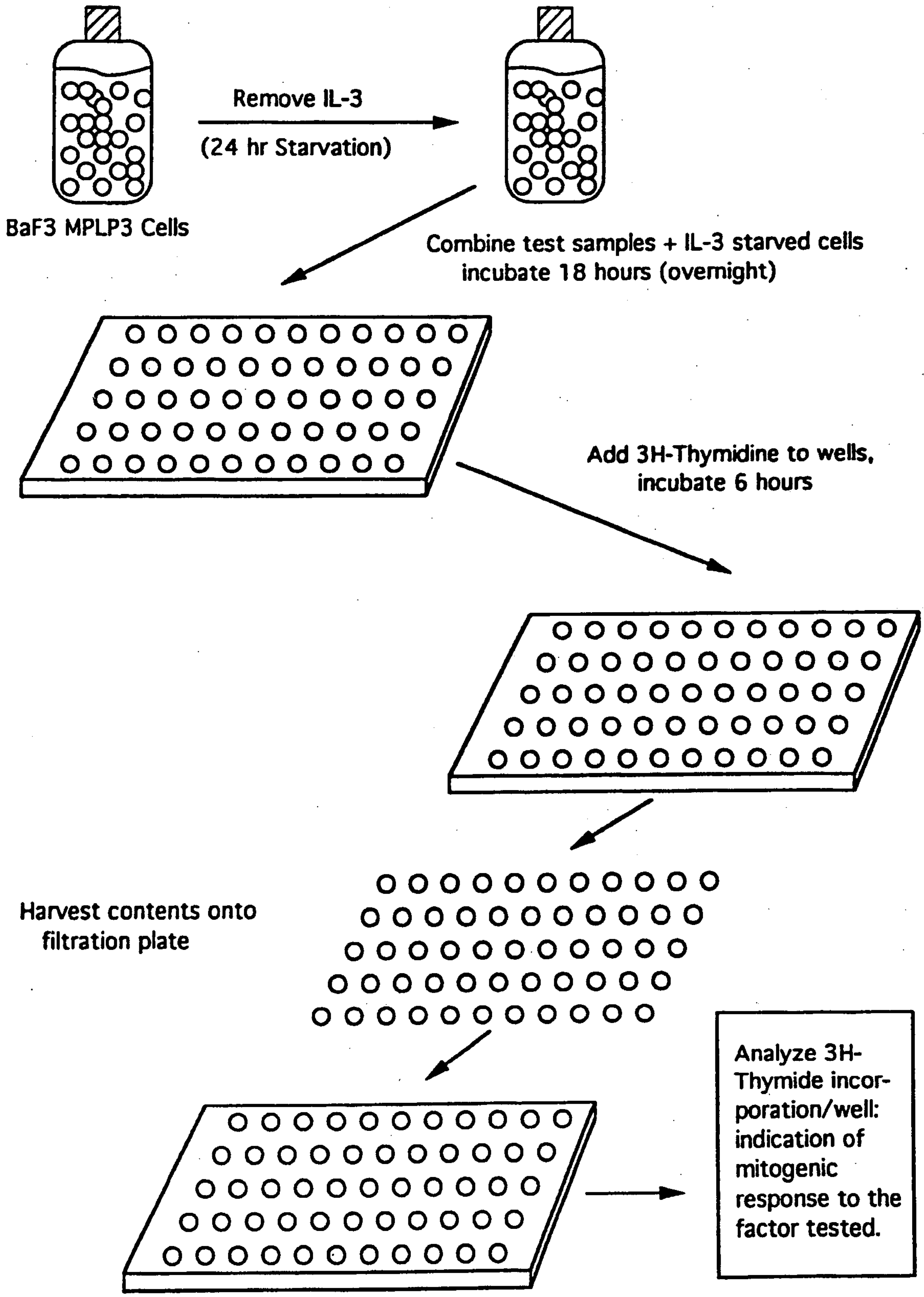

Remove IL-3
(24 hr Starvation)
BaF3 MPLP3 Cells
Combine test samples + IL-3 starved cells
incubate 18 hours (overnight)
Add 3H-Thymidine to wells,
incubate 6 hours
Harvest contents onto
filtration plate
Analyze 3H-Thymide incorporation/well: indication of mitogenic response to the factor tested.

FIG.2

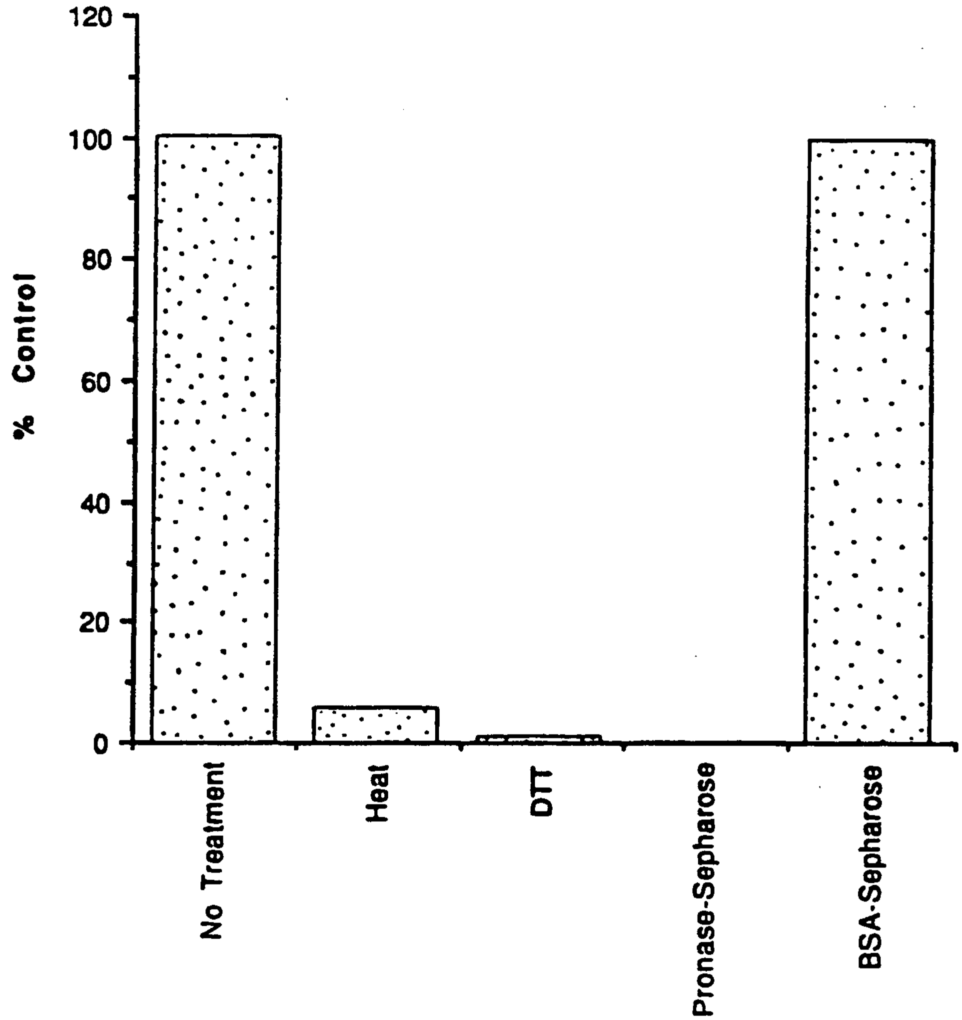
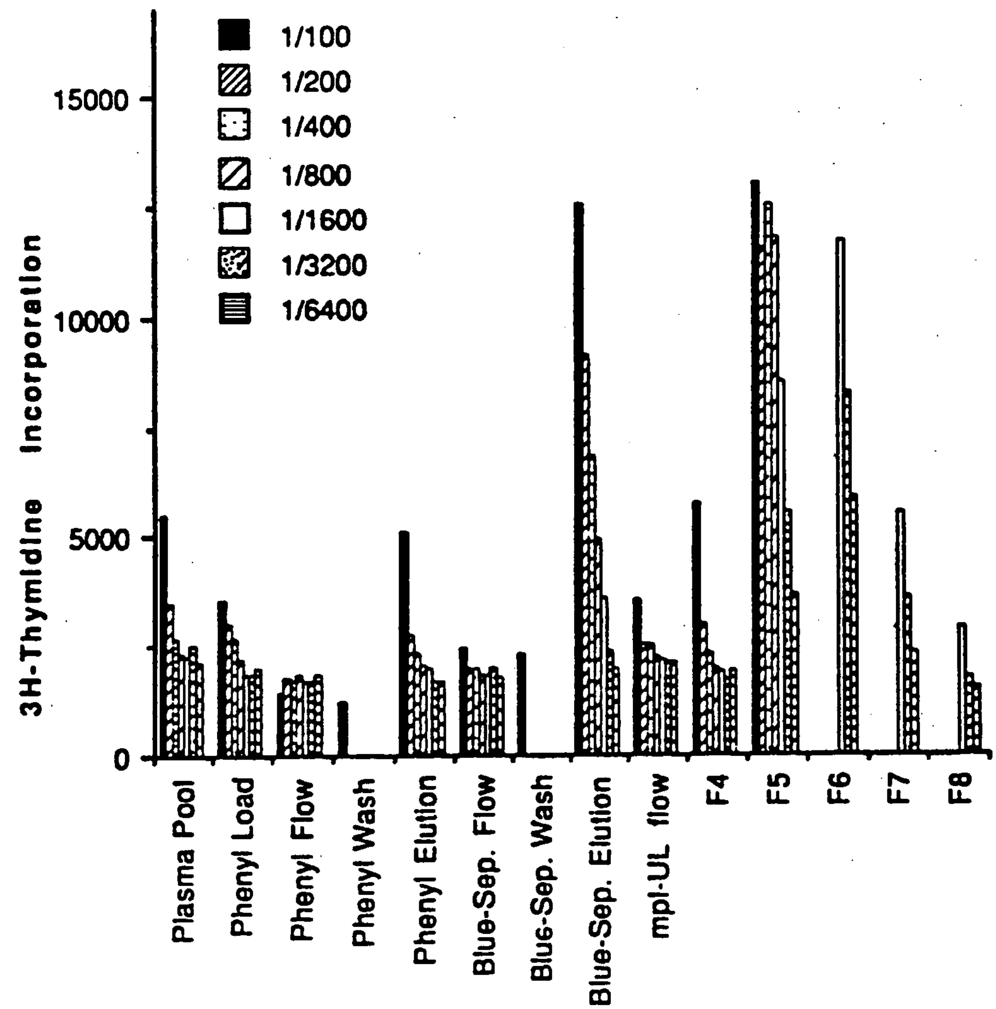

120
100
80
60
40
20
0
% Control
No Treatment
Heat
DTT
Pronase-Sepharose
BSA-Sepharose

FIG.3



1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
3H-Thymidine Incorporation
15000
10000
5000
0
Plasma Pool
Phenyl Load
Phenyl Flow
Phenyl Wash
Phenyl Elution
Blue-Sep. Flow
Blue-Sep. Wash
Blue-Sep. Elution
mpl-UL flow
F4
F5
F6
F7
F8

FIG.4

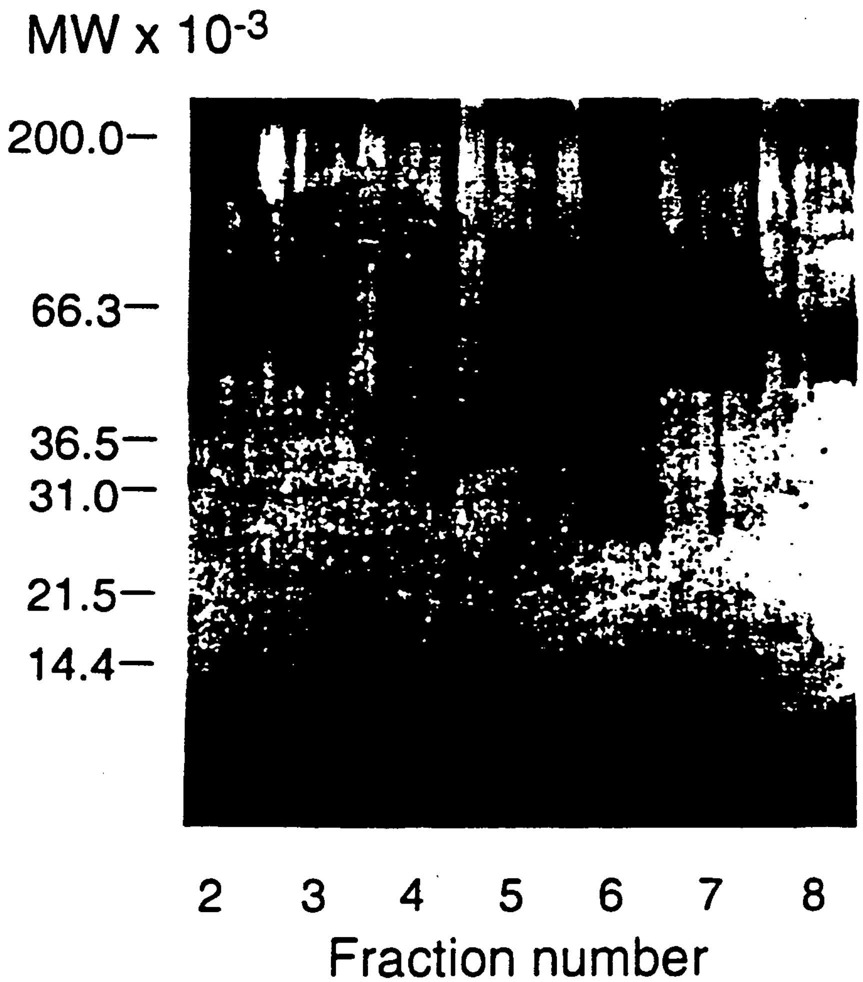


FIG. 5

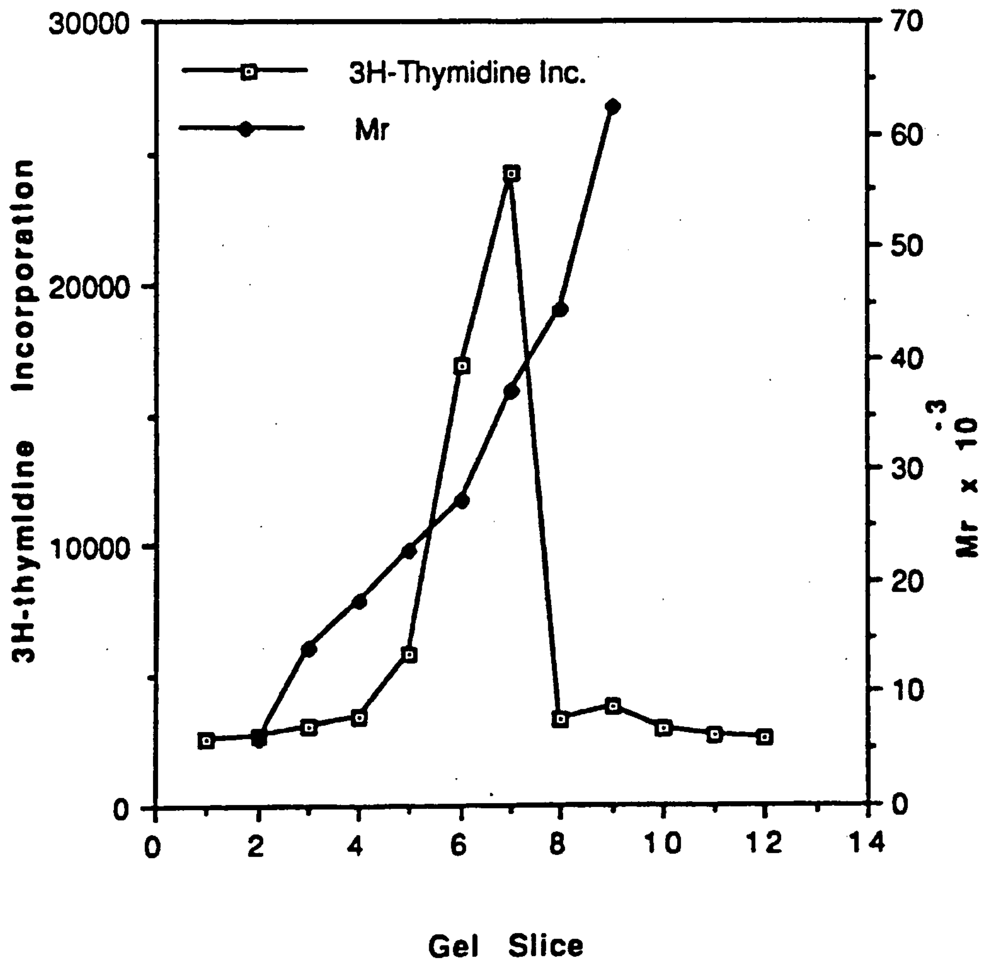
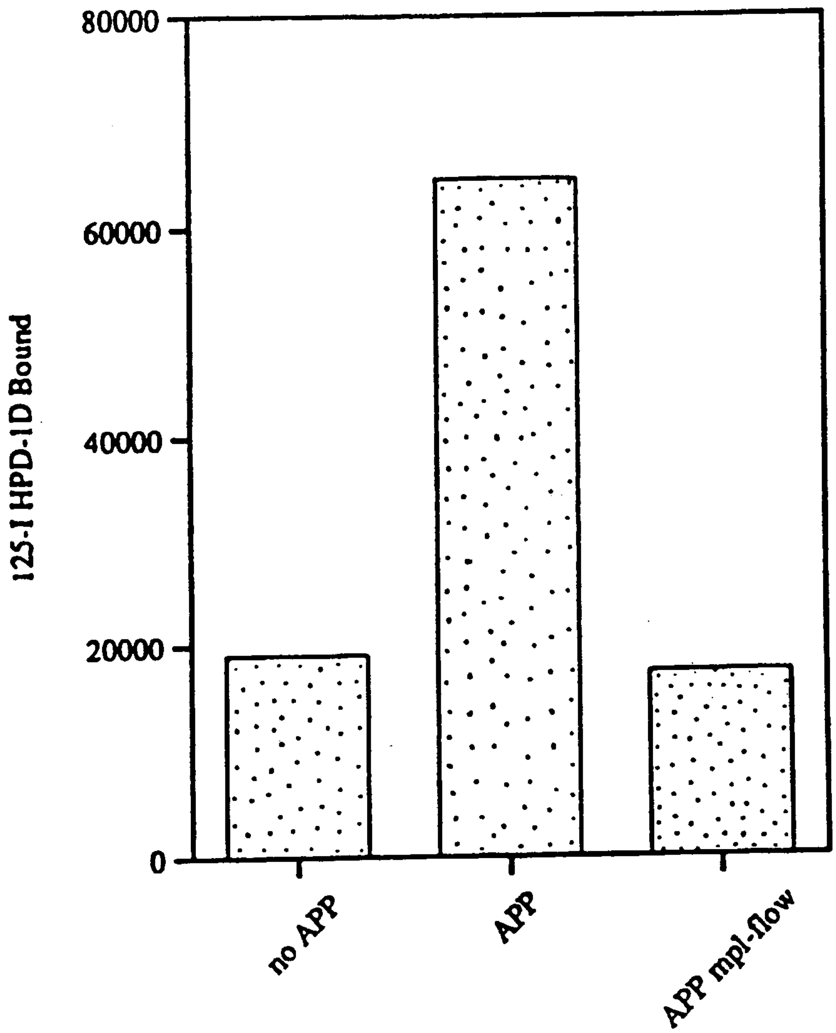
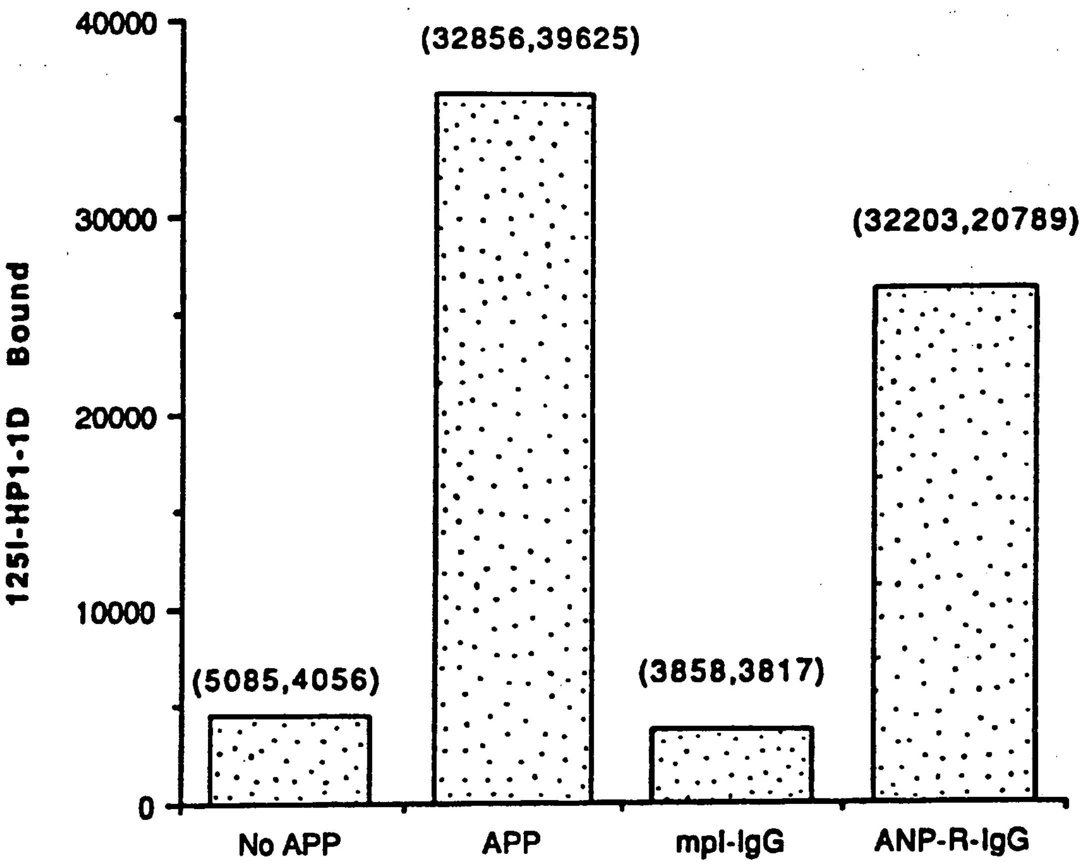

3H-Thymidine Inc.
Mr
3H-thymidine Incorporation
Mr x 10 -3
Gel Slice
30000
20000
10000
0
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14

FIG.6



80000
60000
40000
20000
0
125-I HPD-1D Bound
no APP
APP
APP mpl-flow

FIG.7



40000
30000
20000
10000
0
125I-HP1-1D Bound
(32856,39625)
(32203,20789)
(5085,4056)
(3858,3817)
No APP
APP
mpl-IgG
ANP-R-IgG

FIG.8

```
                                                                                            -10
                                                                          L  L  L  V   V  M  L   L  L  T
  1 GAATTCCTGG AATACCAGCT GACAATGATT TCCTCCTCAT CTTTCAACCT CACCTCTCCT CATCTAAGAA TTGCTCCTCG TGGTCATGCT TCTCCTAACT
    CTTAAGGACC TTATGGTCGA CTGTTACTAA AGGAGGAGTA GAAAGTTGGA GTGGAGAGGA GTAGATTCTT AACGAGGAGC ACCAGTACGA AGAGGATTGA

                                                 10                              20
    A  R  L  T   L  S  S   P  A  P   P  A  C  D   L  R  V   L  S  K   L  L  R  D   S  H  V   L  H  S   R  L
101 GCAAGGCTAA CGCTGTCCAG CCCGGCTCCT CCTGCTTGTG ACCTCCGAGT CCTCAGTAAA CTGCTTCGTG ACTCCCATGT CCTTCACAGC AGACTGGTGA
    CGTTCCGATT GCGACAGGTC GGGCCGAGGA GGACGAACAC TGGAGGCTCA GGAGTCATTT GACGAAGCAC TGAGGGTACA GGAAGTGTCG TCTGACCACT

201 GAACTCCCAA CATTATCCCC TTTATCCGCG TAACTGGTAA GACACCCATA CTCCCAGGAA GACACCATCA CTTCCTCTAA CTCCTTGACC CAATGACTAT
    CTTGAGGGTT GTAATAGGGG AAATAGGCGC ATTGACCATT CTGTGGGTAT GAGGGTCCTT CTGTGGTAGT GAAGGAGATT GAGGAACTGG GTTACTGATA

301 TCTTCCCATA TTGTCCCCAC CTACTGATCA CACTCTCTGA CAAGAATTAT TCTTCACAAT ACAGCCCGCA TTTAAAAGCT CTCGTCTAGA
    AGAAGGGTAT AACAGGGGTG GATGACTAGT GTGAGAGACT GTTCTTAATA AGAAGTGTTA TGTCGGGCGT AAATTTTCGA GAGCAGATCT
```

FIG.9

```
h-ML     1  S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
h-epo    1  A P P R L I C D S R V L E R Y L L E A K E A E N I T T G C A E H C S L N E N I T V P D T K V N F Y A

h-ML    51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S - - S L L G Q L S G Q V R
h-epo   51  W K R M E V G Q Q A V E V W Q G L A L L S E A V L R G Q A L L V N S S Q P W E P L Q L H V D K A V S

h-ML    99  L L - - L G A L Q S L L G T Q - - - L P P Q G R T T A H K D P N A I F L S F Q H L L R G K V R F L -
h-epo  101  G L R S L T T L L R A L G A Q K E A I S P P D A A S A A P L R T I T A D T F R K L F R V Y S N F L R

h-ML   143  - - M L V G G S T L C V R R A P P T T A V P S R T S L V L T L N E L P N R T S G L L E T N F T A S A
h-epo  151  G K L K L Y T G E A C R T G D R

h-ML   191  R T T G S G L L K W Q Q G F R A K I P G L L N Q T S R S L D Q I P G Y L N R I H E L L N G T R G L F

h-ML   241  P G P S R R T L G A P D I S S G T S D T G S L P P N L Q P G Y S P S P T H P P T G Q Y T L F P L P P

h-ML   291  T L P T P V V Q L H P L L P D P S A P T P T P T S P L L N T S Y T H S Q N L S Q E G
```

FIG. 10

```
hML    1   S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
hML2   1   S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
hML3   1   S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
hML4   1   S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E

hML    51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L
hML2   51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L
hML3   51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L
hML4   51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L

hML    101 L G A L Q S L L G T Q L P P Q G R T T A H K D P N A I F L S F Q H L L R G K V R F L M L V G G S T L
hML2   101 L G A L Q S L L G T · · · · Q G R T T A H K D P N A I F L S F Q H L L R G K V R F L M L V G G S T L
hML3   101 L G A L Q S L L G T Q L P P Q G R T T A H K D P N A I F L S F Q H L L R G K · D F W · I V G D K L H
hML4   101 L G A L Q S L L G T · · · · Q G R T T A H K D P N A I F L S F Q H L L R G K · D F W · I V G D K L H

hML    151 C V R R A P P T T A V P S R T S L V L T L N E L P N R T S G L L E T N F T A S A R T T G S G L L K W
hML2   147 C V R R A P P T T A V P S R T S L V L T L N E L P N R T S G L L E T N F T A S A R T T G S G L L K W
hML3   149 C L S Q · · · · · · · · · N Y W L · · · · · · · · · W A S E V A A G I Q S Q D S W S A E P N L Q · ·
hML4   145 C L S Q · · · · · · · · · N Y W L · · · · · · · · · W A S E V A A G I Q S Q D S W S A E P N L Q · ·
```

FIG. 11A

```
hML   201  Q Q G F R A K I P G L L N Q T S R S L D Q I P G Y L N R I H E L L N G T R G L F P G P S R R T L G A
hML2  197  Q Q G F R A K I P G L L N Q T S R S L D Q I P G Y L N R I H E L L N G T R G L F P G P S R R T L G A
hML3  179  V P G P N P R I P . . . E Q D T R T L E W N S W T L S W T L T Q D P R S P G H F L R N I R H R L P A
hML4  175  V P G P N P R I P . . . E Q D T R T L E W N S W T L S W T L T Q D P R S P G H F L R N I R H R L P A

hML   251  P D I S S G T S D T G S L P P N L Q P G Y S P S P T H P P T G Q Y T L F P L P P T L P T P V V Q L H
hML2  247  P D I S S G T S D T G S L P P N L Q P G Y S P S P T H P P T G Q Y T L F P L P P T L P T P V V Q L H
hML3  226  T Q . . . . . . . . . . . P P A W I F S F P . . . . . N P S S Y W T V Y A L P S S . . . . . . . . .
hML4  222  T Q . . . . . . . . . . . P P A W I F S F P . . . . . N P S S Y W T V Y A L P S S . . . . . . . . .

hML   301  P L L P D P S A P T P T P T S P L L N T S Y T H S Q N L S Q E G
hML2  297  P L L P D P S A P T P T P T S P L L N T S Y T H S Q N L S Q E G
hML3  251  T H L A H P C G P A P P P A S . . . . . . . . . . . . . . . . .
hML4  247  T H L A H P C G P A P P P A S . . . . . . . . . . . . . . . . .
```

FIG. 11B

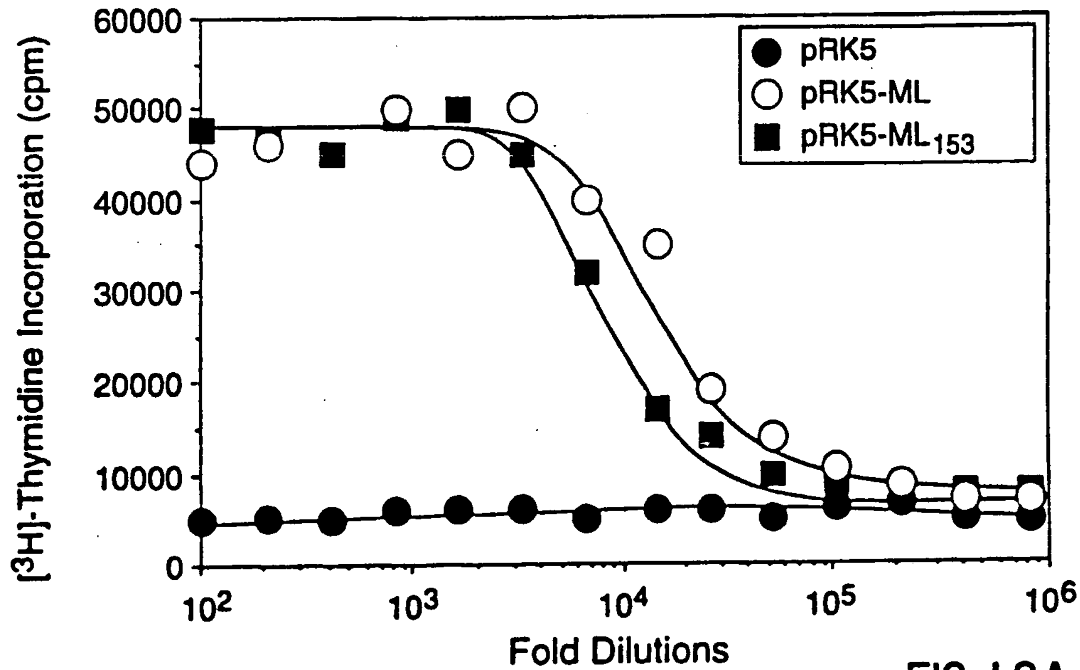


FIG. 12A

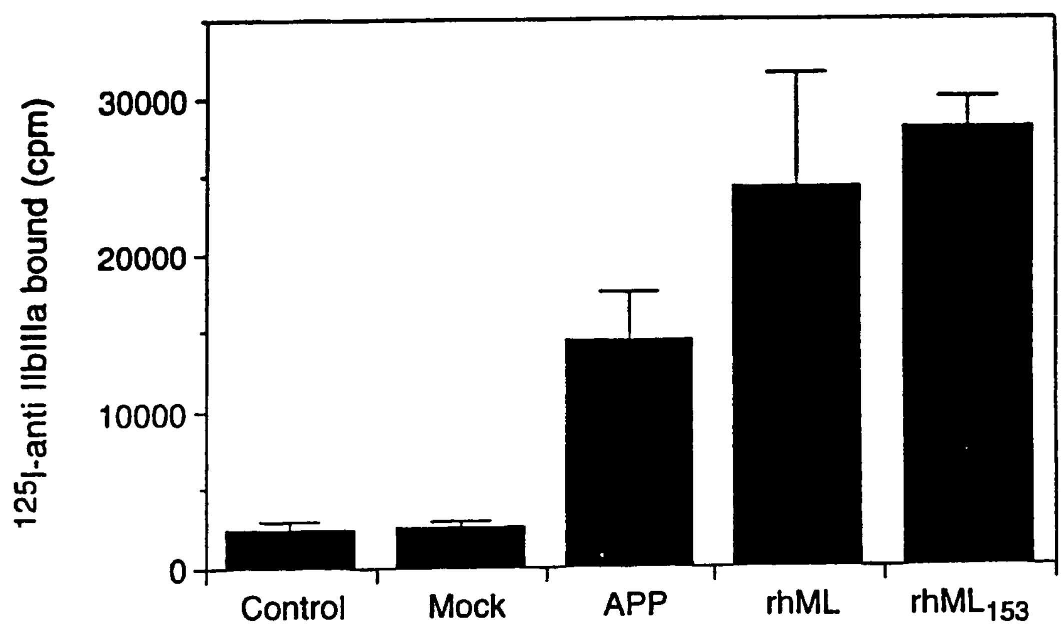


FIG. 12B

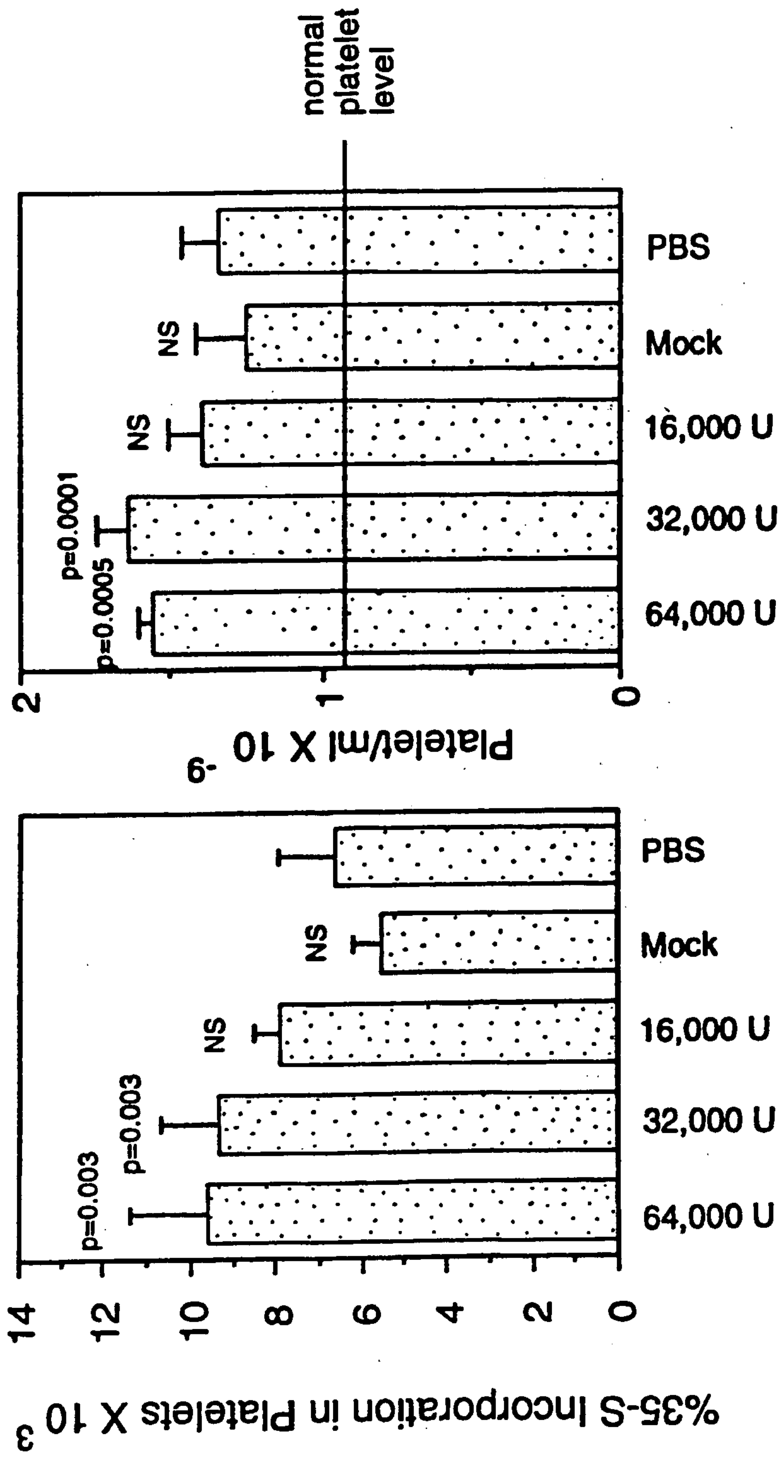

normal platelet level
Platelet/ml X 10 -9
2
1
0
p=0.0001
p=0.0005
NS
NS
PBS
Mock
16,000 U
32,000 U
64,000 U
%35-S Incorporation in Platelets X 10 3
14
12
10
8
6
4
2
0
p=0.003
p=0.003
NS
NS
PBS
Mock
16,000 U
32,000 U
64,000 U

FIG. 12C

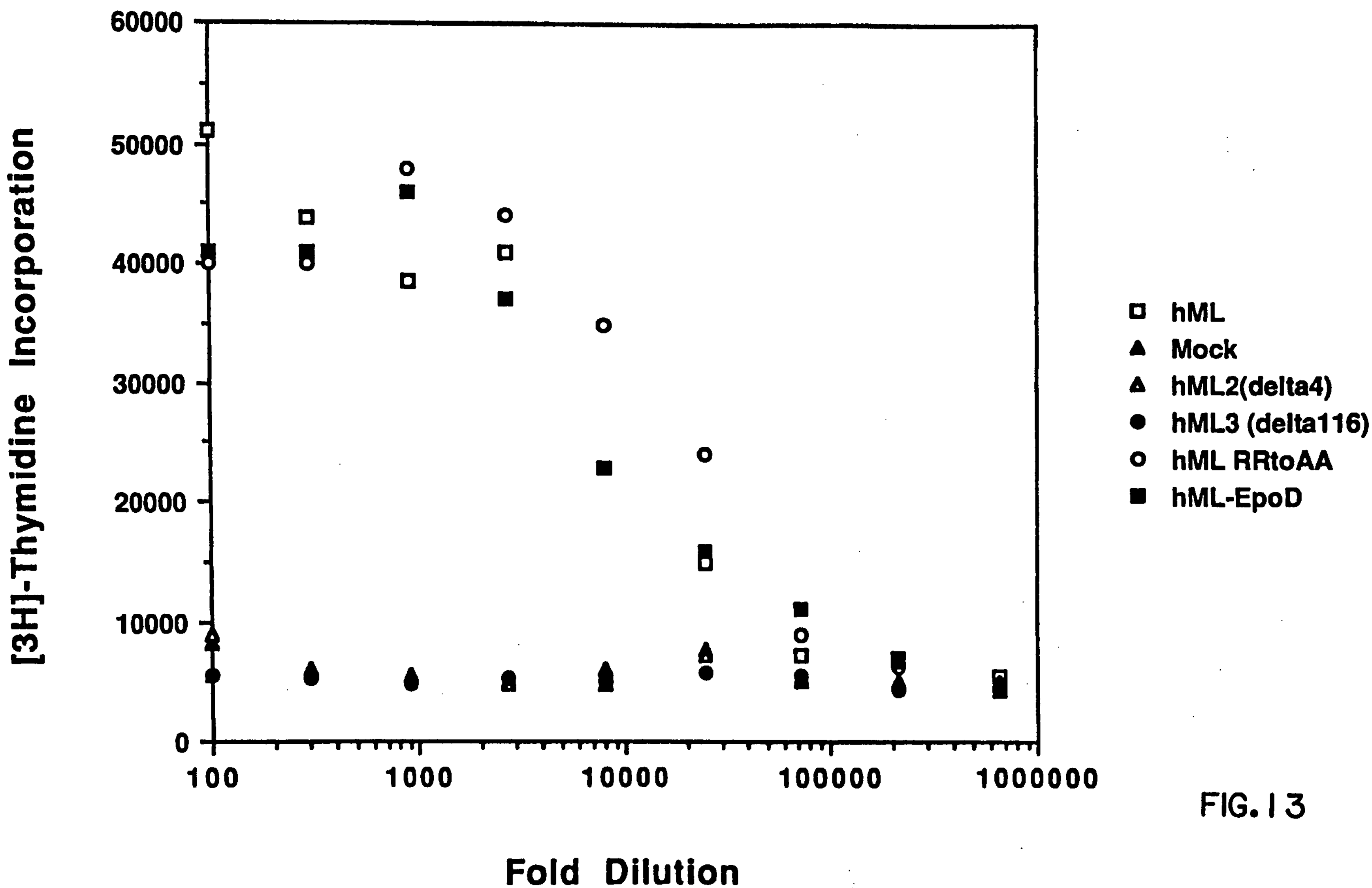

[3H]-Thymidine Incorporation
60000
50000
40000
30000
20000
10000
0
100
1000
10000
100000
1000000
Fold Dilution
hML
Mock
hML2(delta4)
hML3 (delta116)
hML RRtoAA
hML-EpoD

FIG. 13

```
   1 cccagcctcctttctcttgttccctggtcatgcctgcctccctgtctcctgtctctccctcccacacacacccactatcctcccagctatccctacaccc
 101 tccttcctaatcttgggagacatctcgtctggctggacgggaaaattccaggatctaggccacacttctcagcagacatgcccatccttggggaggagga
 201 acaggagagagcctgaggaagttctgggggacagggggatgatgggatcaaggtcaggccaggaagcccctgaggacagagactgtggggagactgggac
 301 tgggaagaaagcaaaggagctagagccagggccaaaggaaaagggggccagcagggaggtatttgcgggggaggtccagcagctgtctttcctaagaca
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 601 gacagtccactcaacccgtccaaaccctttccccataacaccaacccataacaggagatttctctcatgtgggcaatatccgtgttcccacttcgaaagg
 701 gggaatgacaagataggactccctaggggattacagaaagaaaagcaggaaagcaagcatcctgttggatttcagcagcaggtatgatgtccagggaaaa
 801 gaaatttggatagccagggagtgaaaccccaccaatcttaaacaagacctctgtgcttcttccccagcaacacaaatgtcctgccagattcctcctgga
 901 aaaaaacttctgctcctgtcccctccaggtcccaggttgcccatgtccaggaaaagatggatcccctatccaaatcttctccgtggtgtgtgtgggtg
1001 gaggagtggaccctggtccaggcaggggctccagggaagagaaggcgtcacttccgggggccttcaccagtgtctggtggctcccttctctgattgggca
                                                                          < Start of cDNA sequence; Exon 1 >
1101 gaagtggcccaggcaggcgtatgacctgctgctgtggaggggctgtgccccaccgccacatgtctTCCTACCCATCTGCTCCCCAGAGGGCTGCCTGCTG
1201 TGCACTTGGGTCCTGGAGCCCTTCTCCACCCGgtgagtggccagcagggtgtggggttatgtgagggtagaaaggacagcaaagagaaatgggctcccag
1301 ctgggggaggggcaggcaaactggaacctacaggcactgacctttgtcgagaagagtgtagccttcccagaatgggaggagcagggcagagcaggggtag
1401 ggggtggggtgctggtttctgagggactgatcacttacttggtggaatacagcacagccctggctggccctaaggaaaggggacatgagcccagggagaa
1501 aataagagagggagctgcacttagggcttagcaaacacagtagtaagatggacacagccccaatccccattcttagctggtcattcctcgttagcttaag
1601 gttctgaatctggtgctggggaagctgggccaggcaagccagggcgcaaggagagggtaatgggaggagggcccactcatgttgacagacctacaggaaa
```

FIG. 14A

```
1701 tcccaatattgaatcaggtgcaagcctctttgcacaacttgtgaaaggaggaggaagccatgtggggggtcctgtgaaggaaccggaaggggttctgcca

1801 aggggcagggaggcaggtgtgagctatgagacagatatgttagtgggcgcctaagacaaggtaagcccctaaggtgggcatcacccagcaggtgcccgt

1901 tcctgggcagctggtctcaggaaggaagtcccagaactgttagcccatctcttggcctcagataatggagtatttcaggacttggagtccaaagaaaagc

2001 tccagtggctttatgtgtgggggtagatagggaaagaatagaggttaatttctcccataccgccttttaatcctgacctctagtggtcccagttacagct

2101 ttgtgcagttcccctccccagccccactccccaccgcagaagttacccctcaacatattgcgcccgtttgccagttcctcacccaggccctgcatcccat

2201 tttccactctcttctccaggctgaagccacaatactttccttctctatccccatcccagatttctctgacctaacaaccaaggttgctcagaatttaag

2301 gctaattaagatatgtgtgtatacatatcatgtcctgctgctctcagcaggggtaggtggcaccaaatccgtgtccgattcactgaggagtcctgacaaa

2401 aaggagacaccatatgctttcttgctttctttctttctttctttcttttttttttttgagacggagtttcactcttattgcccaggctggagtgcaatg

2501 gtgcgatctcggctcaccacaaacctccgcctcccaggtacaagcgattctcctgtctcagcctcccaagtagcttggattacaggcatgagccaccaca

2601 ccctgctagtttttttgtatttcgtagagccggggtttcaccatgttagtgaggctggtggcgaactcctgacctcaggtgatccacccgccttggactc

2701 ccaaagtgctgggattacaggcatgagccactgcacccggcacaccatatgctttcatcacaagaaaatgtgagagaattcagggctttggcagttccag

2801 gctggtcagcatctcaagccctccccagcatctgttcaccctgccaggcagtctcttcctagaaacttggttaaatgttcactcttcttgctactttcag

      < Exon 2 >
2901 GATAGATTCCTCACCCTTGGCCCGCCTTTGCCCCACCCTACTCTGCCCAGAAGTGCAAGAGCCTAAGCCGCCTCCATGGCCCCAGGAAGGATTCAGGGGA

   1                                               MetGluLeuThrG
3001 GAGGCCCCAAACAGGGAGCCACGCCAGCCAGACACCCCGGCCAGAATGGAGCTGACTGgtgagaacacacctgaggggctagggccatatggaaacatga

3101 cagaaggggagagagaaaggagacacgctgcagggggcaggaagctgggggaacccattctcccaaaaataaggggtctgaggggtggattccctgggtt
                                                                                             < Exon 3 >
   6                                                                                         luLeuLeuLeu
3201 tcaggtctgggtcctgaatgggaattcctggaataccagctgacaatgatttcctcctcatctttcaacctcacctctcctcatctaagAATTGCTCCTC
```

FIG. 14B

```
   9 ValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSerHisVal
3301 GTGGTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGTGACCTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATG
                     End of signal peptide^

  43  LeuHisSerArgLeu
3401 TCCTTCACAGCAGACTGgtgagaactcccaacattatcccctttatccgcgtaactggtaagacacccatactcccaggaagacaccatcacttcctcta

3501 actccttgacccaatgactattcttcccatattgtccccacctactgatcacactctctgacaagaattattcttcacaatacagcccgcatttaaaagc

3601 tctcgtctagagatagtactcatggaggactagcctgcttattaggctaccatagctctctctatttcagctcccttctcccccaccaatctttttcaa
       < Exon 4 >
  48   SerGlnCysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSerLeuGlyGluTrpLysThrGlnMet
3701 cagAGCCAGTGCCCAGAGGTTCACCCTTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGCTTGGGAGAATGGAAAACCCAGATGgtaagaaagc

3801 catccctaaccttggcttccctaagtcctgtcttcagtttcccactgcttcccatggattctccaacattcttgagctttttaaaaatatctcaccttca

3901 gcttggccaccctaacccaatctacattcacctatgatgatagcctgtggataagatgatggcttgcaggtccaatatgtgaatagatttgaagctgaac

4001 accatgaaaagctggagagaaatcgctcatggccatgcctttgacctattccygttcagtcttcttaaattggcatgaagaagcaagactcatatgtcat

4101 ccacagatgacacaaagctgggaagtaccactaaaataacaaaagactgaatcaagattcaaatcactgaaagactaggtcaaaaacaaggtgaaacaac

4201 agagatataaacttctacatgtgggccgggggctcacgcctgtaatcccagcactttgggaggccgaggcaggcagatcacctgagggcaggagtttgag

4301 agcagcctggccaacatggcgaaaccccgtctctactaagaatacaaaattagccgggcatggtagtgcatgcctgtaatcccagctacttggaaggctg

4401 aagcaggagaatcccttgaacccaggaggtggaggttgtagtgagctgagatcatgccaatgcactccagcctgggtgacaagagcaaaactccgtctca

4501 aaaagaaaaaaaaattctacatgtgtaaattaatgagtaaagtcctattccagctttcaggccacaatgccctgcttccatcatttaagcctctggccct

4601 agcacttcctacgaaaaggatctgagagaattaaattgcccccaaacttaccatgtaacattactgaagctgctattcttaaagctagtaattcttgtct

4701 gtttgatgtttagcatccccattgtggaaatgctcgtacagaactctattccgagtggactacacttaaatatactggcctgaacaccggacatcccccct

4801 gaagacatatgctaatttattaagagggaccatattaaactaacatgtgtctagaaagcagcagcctgaacagaaagagactagaagcatgttttatggg
```

FIG. 14C

```
4901 caatagtttaaaaaactaaaatctatcctcaagaaccctagcgtcccttcttccttcaggactgagtcagggaagaagggcagttcctatgggtcccttc

5001 tagtcctttcttttcatccttatgatcattatggtagagtctcatacctacatttagtttattattattatttgagacggagtctcactctatccc

5101 ccaggctggagtgcagtggcatgatctcaactcactgcaacctcagcctcccggattcaagcgattctcctgcctcagtctcccaagtagctgggattac

5201 aggtgcccaccaccatgcccagctaattttgtatttttggtagagatggggtttcaccatgttggccaggctgatcttgaactcctgacctcaggtgat

5301 ccacctgcctcagcctcccaaagtgctgggattacaggcgtgagccactgcacccagccttcattcagtttaaaaatcaaatgatcctaaggttttgcag

5401 cagaaagagtaaatttgcagcactagaaccaagaggtaaaagctgtaacagggcagatttcagcaacgtaagaaaaaggagctcttctcactgaaacca

5501 agtgtaagaccaggctggactagaggacacgggagtttttgaagcagaggctgatgaccagctgtcgggagactgtgaaggaattcctgccctgggtggg

5601 accttggtcctgtccagttctcagcctgtatgattcactctgctggctactcctaaggctccccacccgcttttagtgtgccctttgaggcagtgcgctt
                                            < Exon 5 >
  77                                        GluGluThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeu
5701 ctctcttccatctctttctcagGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTGACCCTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACTG

 103 GlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGln
5801 GGACCCACTTGCCTCTCATCCCTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTCCTTGGAACCCAGgtaagtcccc

5901 agtcaagggatctgtagaaactgttcttttctgactcagtcccactagaagacctgagggaagaagggctcttccagggagctcaagggcagaagagctg

6001 atctactaagagtgctccctgccagccacaatgcctgggtactggcatcctgtctttcctacttagacaagggaggcctgagatctggccctggtgtttg
                                                   < Exon 6 >
 133                                               LeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIlePheLeuSerPheGlnHisLeuLeuArg
6101 GCCTCAGGACCATCCTCTGCCCTCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATCTTCCTGAGTTTCCAACACCTGCTCCG
                                                   ============^Alternative splice site            ^C for cDNA clone

 158 GlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeuValLeu
6201 AGGAAAGGTGCGTTTCCTGATGCTTGTAGGAGGGTCCACCCTCTGCGTCAGGCGGGCCCCACCCACCACAGCTGTCCCCAGCAGAACCTCTCTAGTCCTC
                                                                             ^End of EPO dormain
 191 ThrLeuAsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThrAlaSerAlaArgThrThrGlySerGlyLeuLeuLysTrpGlnGlnGly
6301 ACACTGAACGAGCTCCCAAACAGGACTTCTGGATTGTTGGAGACAAACTTCACTGCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGG
```

FIG. 14D

```
225   PheArgAlaLysIleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsnArgIleHisGluLeuLeuAsnGlyThr
6401 GATTCAGAGCCAAGATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAACAGGATACACGAACTCTTGAATGGAAC

258  ArgGlyLeuPheProGlyProSerArgArgThrLeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeuGlnPro
6501 TCGTGGACTCTTTCCTGGACCCTCACGCAGGACCCTAGGAGCCCCGGACATTTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCTCCAGCCT

291 GlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeuPheProLeuProProThrLeuProThrProValValGlnLeuHisProLeuLeu
6601 GGATATTCTCCTTCCCCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCCTCTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGC

325   ProAspProSerAlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThrHisSerGlnAsnLeuSerGlnGluGly:STOP
6701 TTCCTGACCCTTCTGCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAATCTGTCTCAGGAAGGGTAAGGTTCTCA

6801 GACACTGCCGACATCAGCATTGTCTCATGTACAGCTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAACTGGACAAGATTTCCTACTTTCTCCTGAAACCC

6901 AAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTGTACATTATAAACCTTCAGAAGCTATTTTTTTAAGCTATCAGCAATAC

7001 TCATCAGAGCAGCTAGCTCTTTGGTCTATTTTCTGCAGAAATTTGCAACTCACTGATTCTCTACATGCTCTTTTTCTGTGATAACTCTGCAAAGGCCTGG

7101 GCTGGCCTGGCAGTTGAACAGAGGGAGAGACTAACCTTGAGTCAGAAAACAGAGAAAGGGTAATTTCCTTTGCTTCAAATTCAAGGCCTTCCAACGCCCC

7201 CATCCCCTTTACTATCATTCTCAGTGGGACTCTGATCCCATATTCTTAACAGATCTTTACTCTTGAGAAATGAATAAGCTTTCTCTCAGaaatgctgtcc
                                                                                                 ^PolyA site

7301 ctatacactagacaaaactgagcctgtataaggaataaatgggagcgccgaaaagctccctaaaaagcaagggaaagatgttcttcgagggtggcaatag

7401 atccccctcaccctgccaccccaaacaaaaaagctaacaggaagccttggagagcctcacaccccaggtaaggctgtgtagacagttcagtaaagacagg

7501 acctggatgtgacagctgagcaaacagctagagctttggcagctcagcaggaggctttgccaggcatggacgcctgcctccctcctgtggaggtcaggag

7601 gaagtgcaggaagtggcatgagtcaggctccttgagctcacacagcaggagaacaagtacaagtcaagtacaagttgaaggctcatttcccagttcccgc

7701 aaatgcatctaaaaagcagctctgtgtgaccaccataaactctgctaggggatctctaaaaaggagtcaggcttatggggctttgcaaataagtgctgcc

7801 ttggtgctcaggaaaaggtttgtgttgcacaaaacacaaattccactgc
```

FIG. 14E

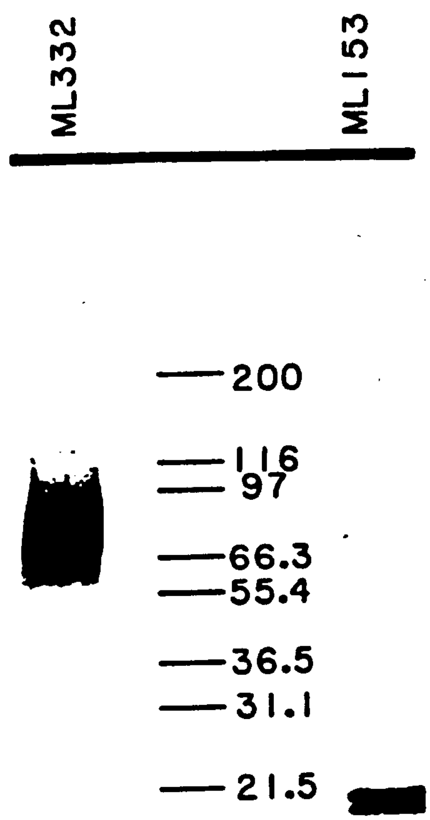


FIG.15

```
  1 GAGTCCTTGG CCCACCTCTC TCCCACCCGA CTCTGCCGAA AGAAGCACAG AAGCTCAAGC CGCCTCCATG GCCCCAGGAA AGATTCAGGG GAGAGGCCCC

                                                -20                      -10
                                            Met GluLeuThrA spLeuLeuLe uAlaAlaMet LeuLeuAlaV alAlaArgLe uThrLeuSer
101 ATACAGGGAG CCACTTCAGT TAGACACCCT GGCCAGAATG GAGCTGACTG ATTTGCTCCT GGCGGCCATG CTTCTTGCAG TGGCAAGACT AACTCTGTCC

                          10                    20                    30
    SerProValA laProAlaCy sAspProArg LeuLeuAsnL ysLeuLeuAr gAspSerHis LeuLeuHisS erArgLeuSe rGlnCysPro AspValAspPro
201 AGCCCCGTAG CTCCTGCCTG TGACCCCAGA CTCCTAAATA AACTGCTGCG TGACTCCCAC CTCCTTCACA GCCGACTGAG TCAGTGTCCC GACGTCGACC

                  40                    50                    60
     LeuSerIl ePro ValLeu LeuProAlaV alAspPheSe rLeuGlyGlu TrpLysThrG lnThrGluGl nSerLysAla GlnAspIleL euGlyAlaVal
301 CTTTGTCTAT CCCTGTTCTG CTGCCTGCTG TGGACTTTAG CCTGGGAGAA TGGAAAAACCC AGACGGAACA GAGCAAGGCA CAGGACATTC TAGGGGCAGT

           70                    80                    90                   100
    SerLeuLeu LeuGluGlyV alMetAlaAl aArgGlyGln LeuGluProS erCysLeuSe rSerLeuLeu GlyGlnLeuS erGlyGlnVa lArgLeuLeu
401 GTCCCTTCTA CTGGAGGGAG TGATGGCAGC ACGAGGACAG TTGGAACCCT CCTGCCTCTC ATCCCTCCTG GGACAGCTTT CTGGGCAGGT TCGCCTCCTC

                         110                   120                   130
    LeuGlyAlaL euGlnGlyLe uLeuGlyThr GlnGlyArgT hrThrAlaHi sLysAspPro AsnAlaLeuP heLeuSerLe uGlnGlnLeu LeuArgGlyLys
501 TTGGGGGCCC TGCAGGGCCT CCTAGGAACC CAGGGCAGGA CCACAGCTCA CAAGGACCCC AATGCCCTCT TCTTGAGCTT GCAACAACTG CTTCGGGGAA

              140                   150                   160
     ValArgPh eLeuLeuLeu ValGluGlyP roThrLeuCy sValArgArg ThrLeuProT hrThrAlaVa lProSerSer ThrSerGlnL euLeuThrLeu
601 AGGTGCGCTT CCTGCTTCTG GTAGAAGGTC CCACCCTCTG TGTCAGACGG ACCCTGCCAA CCACAGCTGT CCCAAGCAGT ACTTCTCAAC TCCTCACACT
```

FIG. 16A

```
                170                                180                                190                                200
     AsnLysPhe ProAsnArgT hrSerGlyLe uLeuGluThr AsnPheSerV alThrAlaAr gThrAlaGly ProGlyLeuL euSerArgLe uGlnGlyPhe
 701 AAACAAGTTC CCAAACAGGA CTTCTGGATT GTTGGAGACG AACTTCAGTG TCACAGCCAG AACTGCTGGC CCTGGACTTC TGAGCAGGCT TCAGGGATTC

                                   210                                220                                230
     ArgValLysI leThrProGl yGlnLeuAsn GlnThrSerA rgSerProVa lGlnIleSer GlyTyrLeuA snArgThrHi sGlyProVal AsnGlyThrHis
 801 AGAGTCAAGA TTACTCCTGG TCAGCTAAAT CAAACCTCCA GGTCCCCAGT CCAAATCTCT GGATACCTGA ACAGGACACA CGGACCTGTG AATGGAACTC

                        240                                250                                260
       GlyLeuPh eAlaGlyThr SerLeuGlnT hrLeuGluAl aSerAspIle SerProGlyA laPheAsnLy sGlySerLeu AlaPheAsnL euGlnGlyGly
 901 ATGGGCTCTT TGCTGGAACC TCACTTCAGA CCCTGGAAGC CTCAGACATC TCGCCCGGAG CTTTCAACAA AGGCTCCCTG GCATTCAACC TCCAGGGTGG

                270                                280                                290                                300
     LeuProPro SerProSerL euAlaProAs pGlyHisThr ProPheProP roSerProAl aLeuProThr ThrHisGlyS erProProGl nLeuHisPro
1001 ACTTCCTCCT TCTCCAAGCC TTGCTCCTGA TGGACACACA CCCTTCCCTC CTTCACCTGC CTTGCCCACC ACCCATGGAT CTCCACCCCA GCTCCACCCC

                                   310                                320                                330
     LeuPheProA spProSerTh rThrMetPro AsnSerThrA laProHisPr oValThrMet TyrProHisP roArgAsnLe uSerGlnGlu Thr
1101 CTGTTTCCTG ACCCTTCCAC CACCATGCCT AACTCTACCG CCCCTCATCC AGTCACAATG TACCCTCATC CCAGGAATTT GTCTCAGGAA ACATAGCGCG

1201 GGCACTGGCC CAGTGAGCGT CTGCAGCTTC TCTCGGGGAC AAGCTTCCCC AGGAAGGCTG AGAGGCAGCT GCATCTGCTC CAGATGTTCT GCTTTCACCT

1301 AAAAGGCCCT GGGGAAGGGA TACACAGCAC TGGAGATTGT AAAATTTTAG GAGCTATTTT TTTTTAACCT ATCAGCAATA TTCATCAGAG CAGCTAGCGA

1401 TCTTTGGTCT ATTTTCGGTA TAAATTTGAA AATCACTAAT TCT
```

FIG. 16B

1 gagtccttggcccacctctctcccacccgactctgccgaaagaagcacagaaagttcaagccgcctcccatggccccaggaaagattcaggggagaggcccc

-20 -10

MetGluLeuThrAsnLeuLeuLeuAlaAlaMetLeuLeuAlaValAlaArgLeuThrLeuSer

101 atacagggagccacttcagttagacaccctggccagaATGGAGCTGACTAATTTGCTCCTGGCGGCCATGCTTCTTGCAGTGGCAAGACTAACTCTGTCC

10 20 30

SerProValAlaProAlaCysAspProArgLeuLeuAsnLysLeuLeuArgAspSerHisLeuLeuHisSerArgLeuSerGlnCysProAspValAspPro

201 AGCCCCGTAGCTCCTGCCTGTGACCCCAGACTCCTAAATAAACTGCTGCGTGACTCCCACCTCCTTCACAGCCGACTGAGTCAGTGTCCCGACGTCGACC

40 50 60

LeuSerIleProValLeuLeuProAlaValAspPheSerLeuGlyGluTrpLysThrGlnThrGluGlnSerLysAlaGlnAspIleLeuGlyAlaVal

301 CTTTGTCTATCCCTGTTCTGCTGCCTGCTGTGGACTTTAGCCTGGGAGAATGGAAAACCCAGACGGAACAGAGCAAGGCACAGGACATTCTAGGGGCAGT

70 80 90 100

SerLeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGluProSerCysLeuSerSerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeu

401 GTCCCTTCTACTGGAGGGAGTGATGGCAGCACGAGGACAGTTGGAACCCTCCTGCCTCTCATCCCTCCTGGGACAGCTTTCTGGGCAGGTTCGCCTCCTC

110 120 130

LeuGlyAlaLeuGlnGlyLeuLeuGlyThrGlnLeuProLeuGlnGlyArgThrThrAlaHisLysAspProAsnAlaLeuPheLeuSerLeuGlnGlnLeu

501 TTGGGGGCCCTGCAGGGCCTCCTAGGAACCCAGCTTCCTCTACAGGGCAGGACCACAGCTCACAAGGACCCCAATGCCCTCTTCTTGAGCTTGCAACAAC

140 150 160

LeuArgGlyLysValArgPheLeuLeuLeuValGluGlyProThrLeuCysValArgArgThrLeuProThrThrAlaValProSerSerThrSerGln

601 TGCTTCGGGGAAAGGTGCGCTTCCTGCTTCTGGTAGAAGGTCCCACCCTCTGTGTCAGACGGACCCTGCCAACCACAGCTGTCCCAAGCAGTACTTCTCA

170 180 190 200

LeuLeuThrLeuAsnLysPheProAsnArgThrSerGlyLeuLeuGluThrAsnPheSerValThrAlaArgThrAlaGlyProGlyLeuLeuSerArg

701 ACTCCTCACACTAAACAAGTTCCCAAACAGGACTTCTGGATTGTTGGAGACGAACTTCAGTGTCACAGCCAGAACTGCTGGCCCTGGACTTCTGAGCAGG

FIG. 17A

```
                               210                                    220                                    230
     LeuGlnGlyPheArgValLysIleThrProGlyGlnLeuAsnGlnThrSerArgSerProValGlnIleSerGlyTyrLeuAsnArgThrHisGlyProVal
 801 CTTCAGGGATTCAGAGTCAAGATTACTCCTGGTCAGCTAAATCAAACCTCCAGGTCCCCAGTCCAAATCTCTGGATACCTGAACAGGACACACGGACCTG

                  240                                    250                                    260
       AsnGlyThrHisGlyLeuPheAlaGlyThrSerLeuGlnThrLeuGluAlaSerAspIleSerProGlyAlaPheAsnLysGlySerLeuAlaPheAsn
 901 TGAATGGAACTCATGGGCTCTTTGCTGGAACCTCACTTCAGACCCTGGAAGCCTCAGACATCTCGCCCGGAGCTTTCAACAAAGGCTCCCTGGCATTCAA

          270                                    280                                    290                                    300
      LeuGlnGlyGlyLeuProProSerProSerLeuAlaProAspGlyHisThrProPheProProSerProAlaLeuProThrThrHisGlySerProPro
1001 CCTCCAGGGTGGACTTCCTCCTTCTCCAAGCCTTGCTCCTGATGGACACACACCCTTCCCTCCTTCACCTGCCTTGCCCACCACCCATGGATCTCCACCC

                               310                                    320                                    330
     GlnLeuHisProLeuPheProAspProSerThrThrMetProAsnSerThrAlaProHisProValThrMetTyrProHisProArgAsnLeuSerGlnGlu
1101 CAGCTCCACCCCCTGTTTCCTGACCCTTCCACCACCATGCCTAACTCTACCGCCCCTCATCCAGTCACAATGTACCCTCATCCCAGGAATTTGTCTCAGG

       Thr
1201 AAACATAGcgcgggcactggcccagtgagcgtctgcagcttctctcggggacaagcttccccaggaaggctgagaggcagctgcatctgctccagatgtt

1301 ctgctttcacctaaaaggccctggggaagggatacacagcactggagattgtaaaattttaggagctatttttttttaacctatcagcaatattcatcag

1401 agcagctagcgatctttggtctattttcggtataaatttgaaaatcactaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

1501 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

FIG. 17B

```
hML3    1  S P A P P A C D L R V L S K L L R D S H V L H S R L S Q C P E V H P L P T P V L L P A V D F S L G E
mML3    1  S P V A P A C D P R L L N K L L R D S H L L H S R L S Q C P D V D P L S I P V L L P A V D F S L G E

hML3   51  W K T Q M E E T K A Q D I L G A V T L L L E G V M A A R G Q L G P T C L S S L L G Q L S G Q V R L L
mML3   51  W K T Q T E Q S K A Q D I L G A V S L L L E G V M A A R G Q L E P S C L S S L L G Q L S G Q V R L L

hML3  101  L G A L Q S L L G T Q L P P Q G R T T A H K D P N A I F L S F Q H L L R G K D F W I V G D K L H C L
mML3  101  L G A L Q G L L G T Q L P L Q G R T T A H K D P N A L F L S L Q Q L L R G K D F W I V G D E L Q C H

hML3  151  S Q N Y W L W A S E V A A G I Q S Q D · S W S A E P N L Q V P G P N P R I P E Q D T R T L E W Ñ S W
mML3  151  S Q N C W P W T S E Q A S G I Q S Q D Y S W S A K S N L Q V P S P N L W I P E Q D T R T C E W N S W

hML3  200  T L S W T L T Q D P R S P G H F L R N I R H R L P A T Q P P A W I F S F P N P S S Y W T V Y A L P S
mML3  201  A L C W N L T S D P G S L R H L A R S F Q Q R L P G I Q P P G W T S S F S K P C S

hML3  250  S T H L A H P C G P A P P P A S
```

FIG. 18

```
                                10                                      20                                      30
    SerProAlaProProAlaCysAspProArgLeuLeuAsnLysLeuLeuArgAspSerHisValLeuHisGlyArgLeuSerGlnCysProAspIleAsnPro
  1 AGCCCGGCTCCTCCTGCCTGTGACCCCCGACTCCTAAATAAACTGCTTCGTGACTCCCATGTCCTTCACGGCAGACTGAGCCAGTGCCCAGACATTAACC

                    40                                      50                                      60
     LeuSerThrProValLeuLeuProAlaValAspPheThrLeuGlyGluTrpLysThrGlnThrGluGlnThrLysAlaGlnAspValLeuGlyAlaThr
101 CTTTGTCCACACCTGTCCTGCTGCCTGCTGTGGACTTCACCTTGGGAGAATGGAAAACCCAGACGGAGCAGACAAAGGCACAGGATGTCCTGGGAGCCAC

        70                                      80                                      90                                     100
    ThrLeuLeuLeuGluAlaValMetThrAlaArgGlyGlnValGlyProProCysLeuSerSerLeuLeuValGlnLeuSerGlyGlnValArgLeuLeu
201 AACCCTTCTGCTGGAGGCAGTGATGACAGCACGGGGACAAGTGGGACCCCCTTGCCTCTCATCCCTGCTGGTGCAGCTTTCTGGACAGGTTCGCCTCCTC

                                 110                                     120                                     130
    LeuGlyAlaLeuGlnAspLeuLeuGlyMetGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProSerAlaIlePheLeuAsnPheGlnGlnLeu
301 CTCGGGGCCCTGCAGGACCTCCTTGGAATGCAGCTTCCTCCACAGGGAAGGACCACAGCTCACAAGGATCCCAGTGCCATCTTCCTGAACTTCCAACAAC

                     140                                     150                                     160
     LeuArgGlyLysValArgPheLeuLeuLeuValValGlyProSerLeuCysAlaLysArgAlaProProAlaIleAlaValProSerSerThrSerPro
401 TGCTCCGAGGAAAGGTGCGTTTCCTGCTCCTTGTAGTGGGGCCCTCCCTCTGTGCCAAGAGGGCCCCACCCGCCATAGCTGTCCCGAGCAGCACCTCTCC
```

FIG.20A

```
     170                                180                                190                                200
     PheHisThrLeuAsnLysLeuProAsnArgThrSerGlyLeuLeuGluThrAsnSerSerIleSerAlaArgThrThrGlySerGlyPheLeuLysArg
501  ATTCCACACACTGAACAAGCTCCCAAACAGGACCTCTGGATTGTTGGAGACAAACTCCAGTATCTCAGCCAGAACTACTGGCTCTGGATTTCTCAAGAGG

                        210                                220                                230
     LeuGlnAlaPheArgAlaLysIleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyHisGlnAsnGlyThrHisGlyProLeuSer
601  CTGCAGGCATTCAGAGCCAAGATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTAGACCAAATCCCTGGACACCAGAATGGGACACACGGACCCTTGA

                 240                                250                                260
     GlyIleHisGlyLeuPheProGlyProGlnProGlyAlaLeuGlyAlaProAspIleProProAlaThrSerGlyMetGlySerArgProThrTyrLeu
701  GTGGAATTCATGGACTCTTTCCTGGACCCCAACCCGGGGCCCTCGGAGCTCCAGACATTCCTCCAGCAACTTCAGGCATGGGCTCCCGGCCAACCTACCT

     270                                280                                290                                300
     GlnProGlyGluSerProSerProAlaHisProSerProGlyArgTyrThrLeuPheSerProSerProThrSerProSerProThrValGlnLeuGln
801  CCAGCCTGGAGAGTCTCCTTCCCCAGCTCACCCTTCTCCTGGACGATACACTCTCTTCTCTCCTTCACCCACCTCGCCCTCCCCCACAGTCCAGCTCCAG

                        310                                320                                330
     ProLeuLeuProAspProSerAlaIleThrProAsnSerThrSerProLeuLeuPheAlaAlaHisProHisPheGlnAsnLeuSerGlnGluGlu
901  CCTCTGCTTCCTGACCCCTCTGCGATCACACCCAACTCTACCAGTCCTCTTCTATTTGCAGCTCACCCTCATTTCCAGAACCTGTCTCAGGAAGAGTAAG

1001 GTGCTCAGACCCTGCCAACTTCAGCA
```

FIG.20B

```
                                        10                                        20                                        30
   SerProAlaProProAlaCysAspProArgLeuLeuAsnLysLeuLeuArgAspSerHisValLeuHisGlyArgLeuSerGlnCysProAspIleAsnPro
1  AGCCCGGCTCCTCCTGCCTGTGACCCCCGACTCCTAAATAAACTGCTTCGTGACTCCCATGTCCTTCACGGCAGACTGAGCCAGTGCCCAGACATTAACC

                    40                                        50                                        60
    LeuSerThrProValLeuLeuProAlaValAspPheThrLeuGlyGluTrpLysThrGlnThrGluGlnThrLysAlaGlnAspValLeuGlyAlaThr
101 CTTTGTCCACACCTGTCCTGCTGCCTGCTGTGGACTTCACCTTGGGAGAATGGAAAACCCAGACGGAGCAGACAAAGGCACAGGATGTCCTGGGAGCCAC

     70                                        80                                        90                                        100
    ThrLeuLeuLeuGluAlaValMetThrAlaArgGlyGlnValGlyProProCysLeuSerSerLeuLeuValGlnLeuSerGlyGlnValArgLeuLeu
201 AACCCTTCTGCTGGAGGCAGTGATGACAGCACGGGGACAAGTGGACCCCCTTGCCTCTCATCCCTGCTGGTGCAGCTTTCTGGACAGGTTCGCCTCCTC

                                        110                                       120                                       130
    LeuGlyAlaLeuGlnAspLeuLeuGlyMetGlnGlyArgThrThrAlaHisLysAspProSerAlaIlePheLeuAsnPheGlnGlnLeuLeuArgGlyLys
301 CTCGGGGCCCTGCAGGACCTCCTTGGAATGCAGGGAAGGACCACAGCTCACAAGGATCCCAGTGCCATCTTCCTGAACTTCCAACAACTGCTCCGAGGAA

                    140                                       150                                       160
    ValArgPheLeuLeuLeuValValGlyProSerLeuCysAlaLysArgAlaProProAlaIleAlaValProSerSerThrSerProPheHisThrLeu
401 AGGTGCGTTTCCTGCTCCTTGTAGTGGGGCCCTCCCTCTGTGCCAAGAGGGCCCCACCCGCCATAGCTGTCCCGAGCAGCACCTCTCCATTCCACACACT
```

FIG.21A

```
         170                                180                                190                                200
     AsnLysLeuProAsnArgThrSerGlyLeuLeuGluThrAsnSerSerIleSerAlaArgThrThrGlySerGlyPheLeuLysArgLeuGlnAlaPhe
 501 GAACAAGCTCCCAAACAGGACCTCTGGATTGTTGGAGACAAACTCCAGTATCTCAGCCAGAACTACTGGCTCTGGATTTCTCAAGAGGCTGCAGGCATTC

                        210                                220                                230
     ArgAlaLysIleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyHisGlnAsnGlyThrHisGlyProLeuSerGlyIleHisGly
 601 AGAGCCAAGATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTAGACCAAATCCCTGGACACCAGAATGGGACACACGGACCCTTGAGTGGAATTCATG

                   240                                250                                260
     LeuPheProGlyProGlnProGlyAlaLeuGlyAlaProAspIleProProAlaThrSerGlyMetGlySerArgProThrTyrLeuGlnProGlyGlu
 701 GACTCTTTCCTGGACCCCAACCCGGGGCCCTCGGAGCTCCAGACATTCCTCCAGCAACTTCAGGCATGGGCTCCCGGCCAACCTACCTCCAGCCTGGAGA

         270                                280                                290                                300
     SerProSerProAlaHisProSerProGlyArgTyrThrLeuPheSerProSerProThrSerProSerProThrValGlnLeuGlnProLeuLeuPro
 801 GTCTCCTTCCCCAGCTCACCCTTCTCCTGGACGATACACTCTCTTCTCTCCTTCACCCACCTCGCCCTCCCCCACAGTCCAGCTCCAGCCTCTGCTTCCT

                        310                                320
     AspProSerAlaIleThrProAsnSerThrSerProLeuLeuPheAlaAlaHisProHisPheGlnAsnLeuSerGlnGluGlu
 901 GACCCCTCTGCGATCACACCCAACTCTACCAGTCCTCTTCTATTTGCAGCTCACCCTCATTTCCAGAACCTGTCTCAGGAAGAGTAAGGTGCTCAGACCC

1001 TGCCAACTTCAGCA
```

FIG.21B

pML	1	SPAPPACDPRLLNKLLRDSHVLHGRLSQCPDINPLSTPVLLPAVDFTLGE
pML2	1	SPAPPACDPRLLNKLLRDSHVLHGRLSQCPDINPLSTPVLLPAVDFTLGE
pML	51	WKTQTEQTKAQDVLGATTLLLEAVMTARGQVGPPCLSSLLVQLSGQVRLL
pML2	51	WKTQTEQTKAQDVLGATTLLLEAVMTARGQVGPPCLSSLLVQLSGQVRLL
pML	101	LGALQDLLGMQLPPQGRTTAHKDPSAIFLNFQQLLRGKVRFLLLVVGPSL
pML2	101	LGALQDLLGM....QGRTTAHKDPSAIFLNFQQLLRGKVRFLLLVVGPSL
pML	151	CAKRAPPAIAVPSSTSPFHTLNKLPNRTSGLLETNSSISARTTGSGFLKR
pML2	147	CAKRAPPAIAVPSSTSPFHTLNKLPNRTSGLLETNSSISARTTGSGFLKR
pML	201	LQAFRAKIPGLLNQTSRSLDQIPGHQNGTHGPLSGIHGLFPGPQPGALGA
pML2	197	LQAFRAKIPGLLNQTSRSLDQIPGHQNGTHGPLSGIHGLFPGPQPGALGA
pML	251	PDIPPATSGMGSRPTYLQPGESPSPAHPSPGRYTLFSPSPTSPSPTVQLQ
pML2	247	PDIPPATSGMGSRPTYLQPGESPSPAHPSPGRYTLFSPSPTSPSPTVQLQ
pML	301	PLLPDPSAITPNSTSPLLFAAHPHFQNLSQEE
pML2	297	PLLPDPSAITPNSTSPLLFAAHPHFQNLSQEE

FIG.22

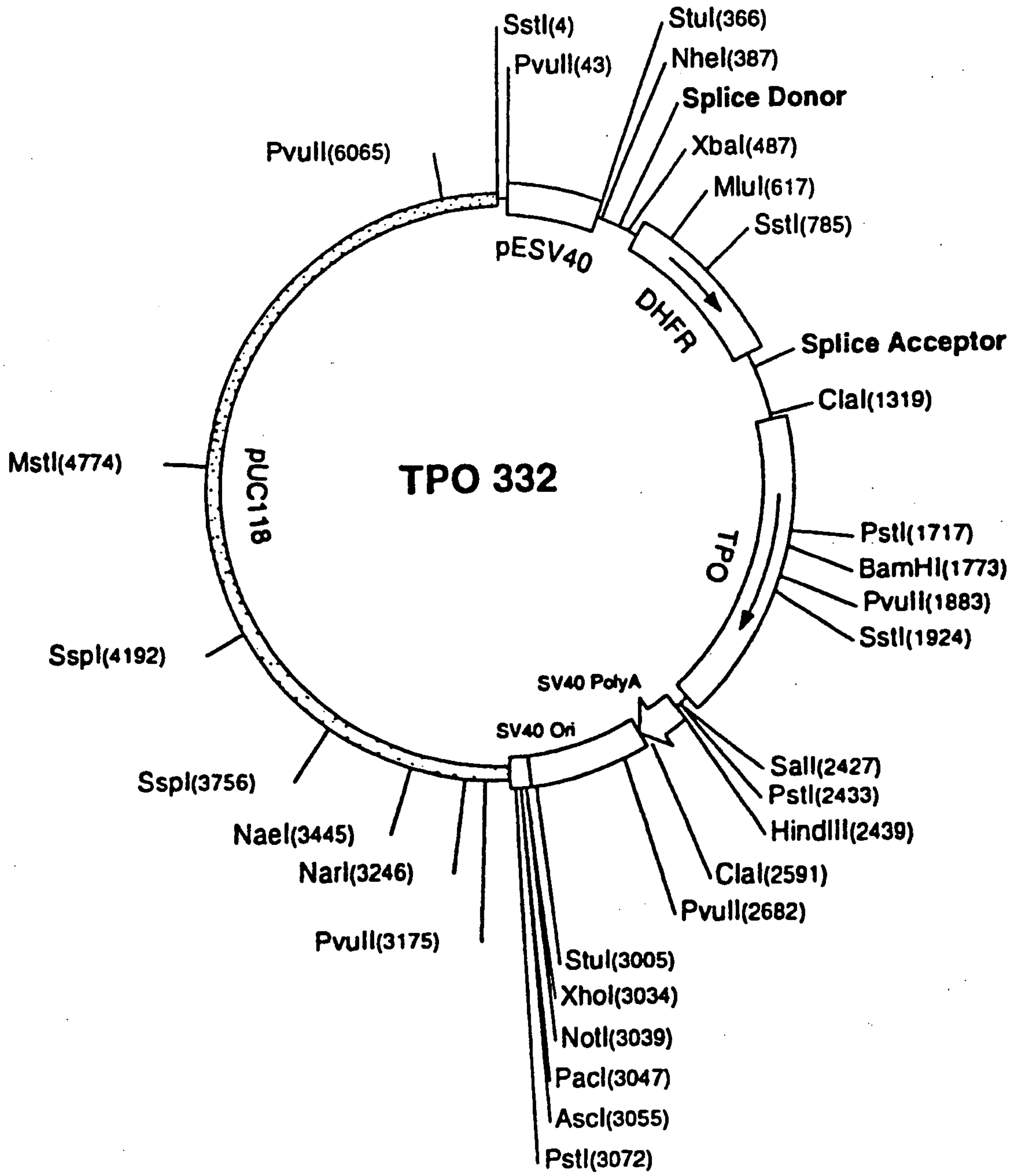

SstI(4)
PvuII(43)
StuI(366)
NheI(387)
Splice Donor
XbaI(487)
MluI(617)
SstI(785)
Splice Acceptor
ClaI(1319)
PstI(1717)
BamHI(1773)
PvuII(1883)
SstI(1924)
SalI(2427)
PstI(2433)
HindIII(2439)
ClaI(2591)
PvuII(2682)
StuI(3005)
XhoI(3034)
NotI(3039)
PacI(3047)
AscI(3055)
PstI(3072)
PvuII(3175)
NarI(3246)
NaeI(3445)
SspI(3756)
SspI(4192)
MstI(4774)
PvuII(6065)
pESV40
DHFR
TPO
SV40 PolyA
SV40 Ori
pUC118
TPO 332

FIG.23

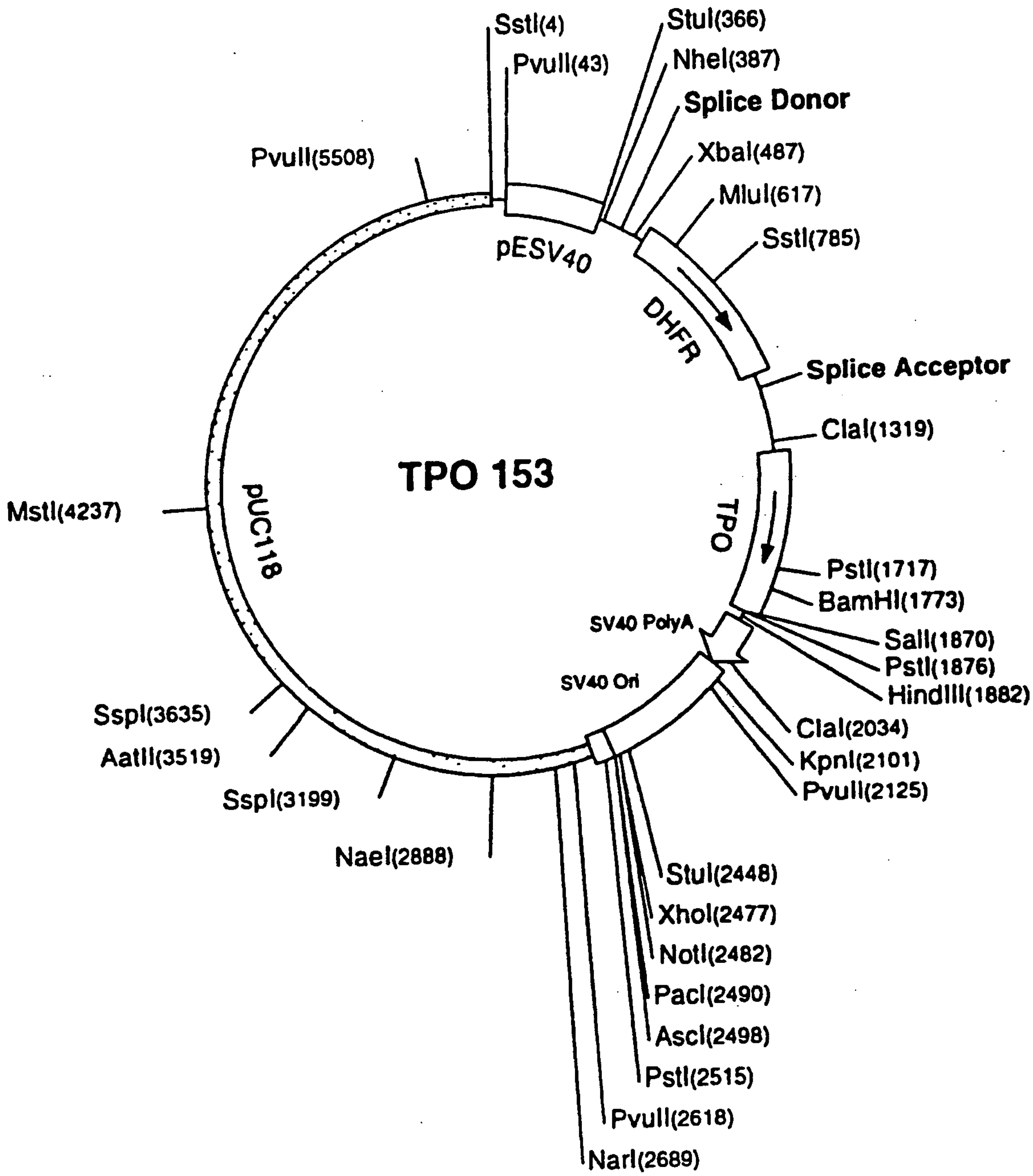

SstI(4)
PvuII(43)
StuI(366)
NheI(387)
Splice Donor
XbaI(487)
MluI(617)
SstI(785)
PvuII(5508)
pESV40
DHFR
Splice Acceptor
ClaI(1319)
TPO 153
TPO
MstI(4237)
pUC118
PstI(1717)
BamHI(1773)
SV40 PolyA
SalI(1870)
PstI(1876)
HindIII(1882)
SV40 Ori
SspI(3635)
ClaI(2034)
AatII(3519)
KpnI(2101)
SspI(3199)
PvuII(2125)
NaeI(2888)
StuI(2448)
XhoI(2477)
NotI(2482)
PacI(2490)
AscI(2498)
PstI(2515)
PvuII(2618)
NarI(2689)

FIG.24

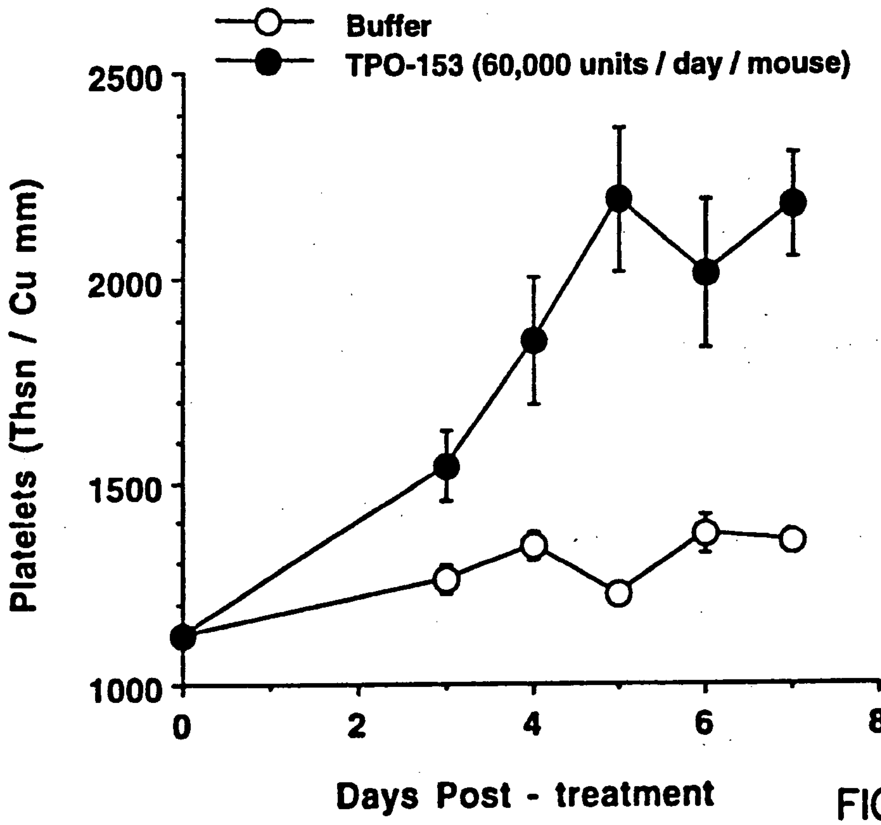


FIG.25A

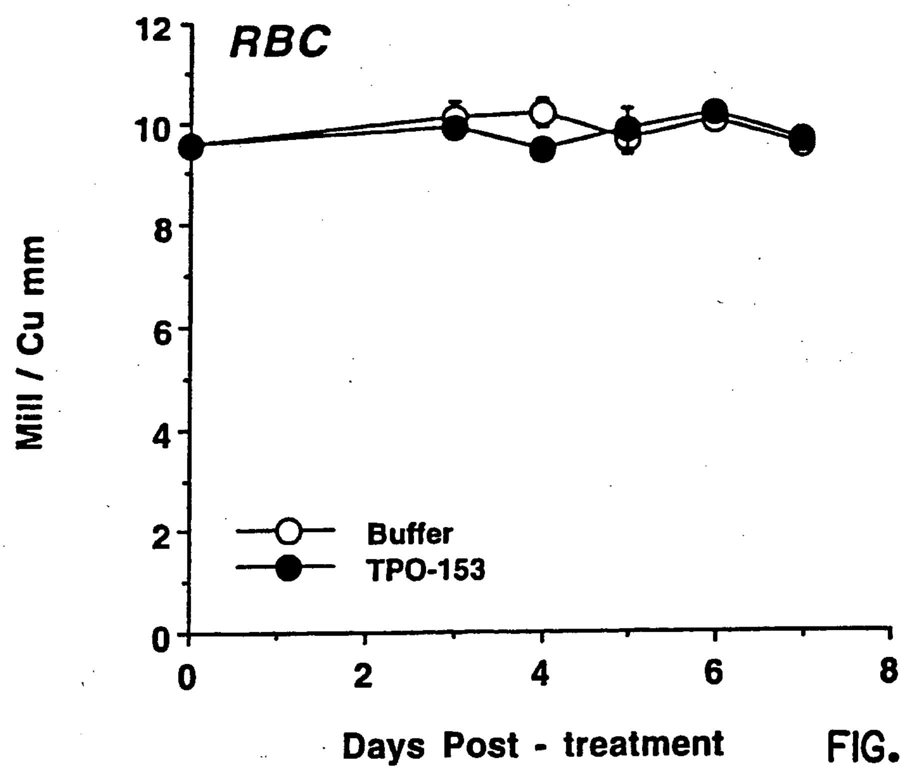


FIG.25B

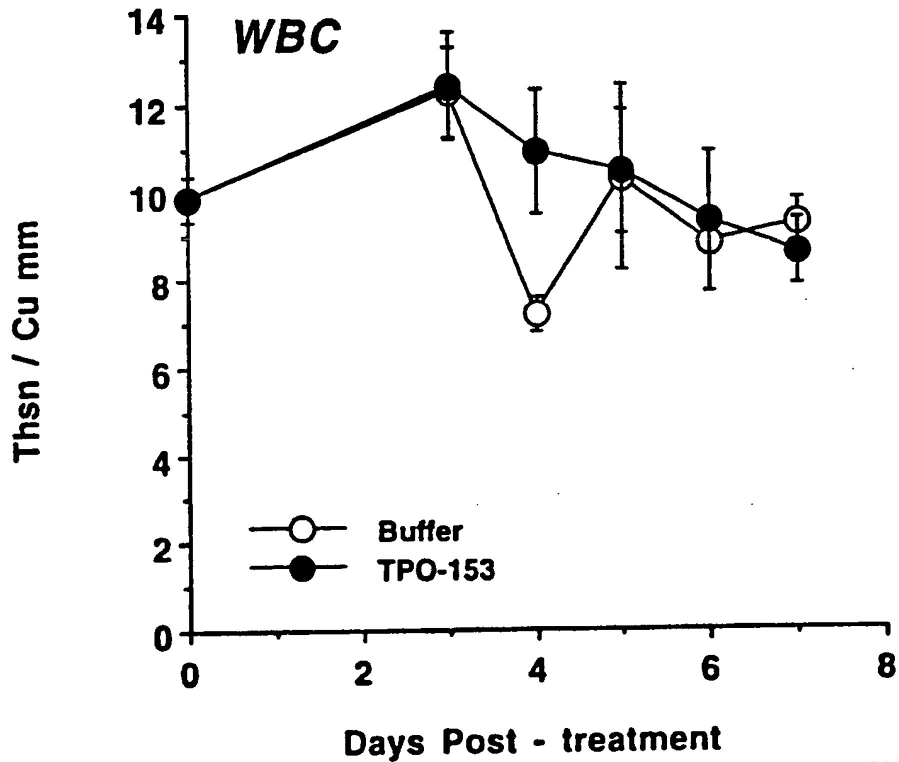


FIG.25C

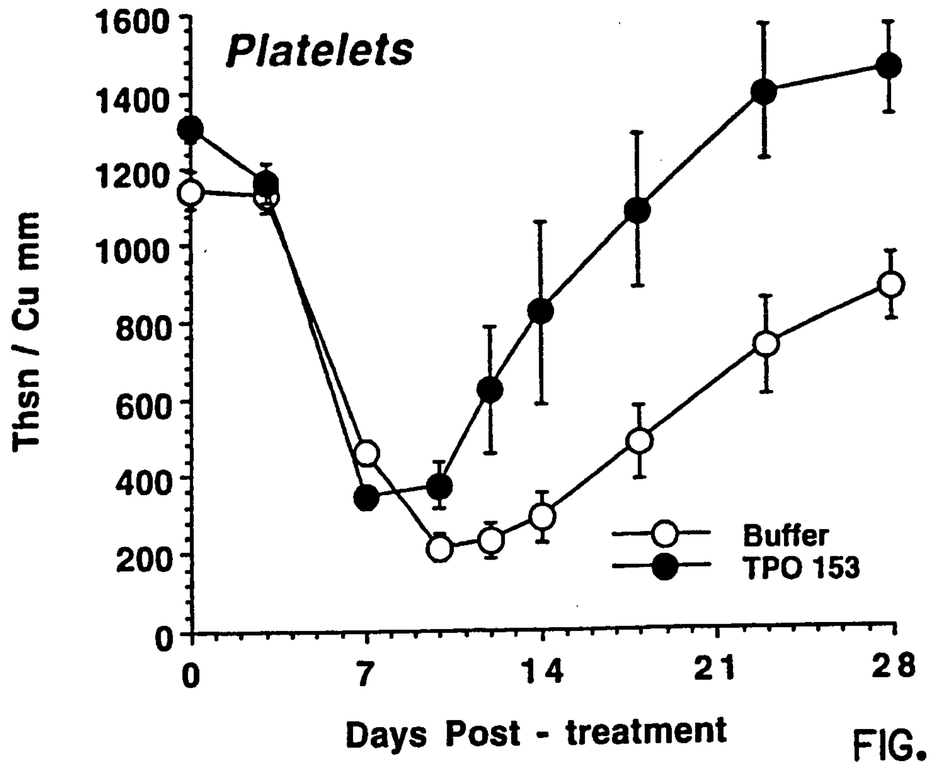


FIG.26A

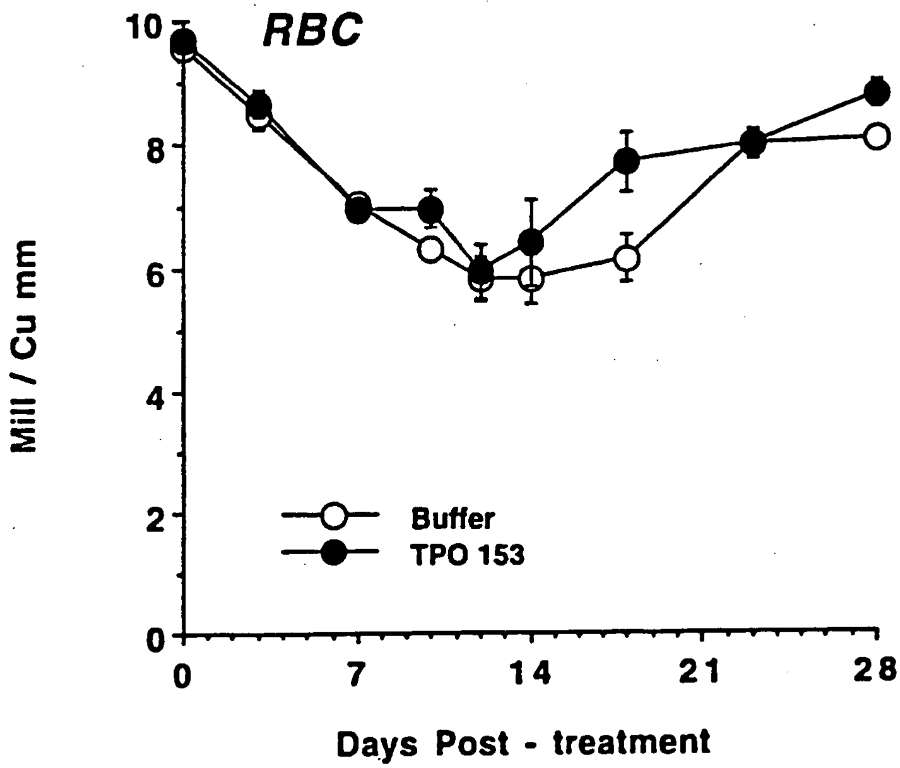


FIG.26B

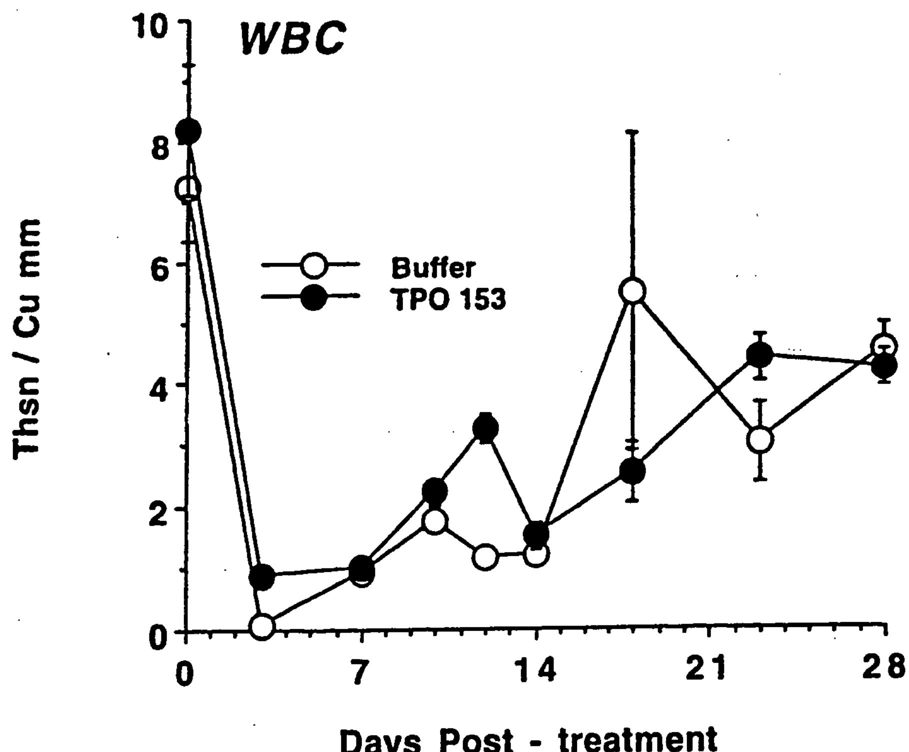


FIG.26C

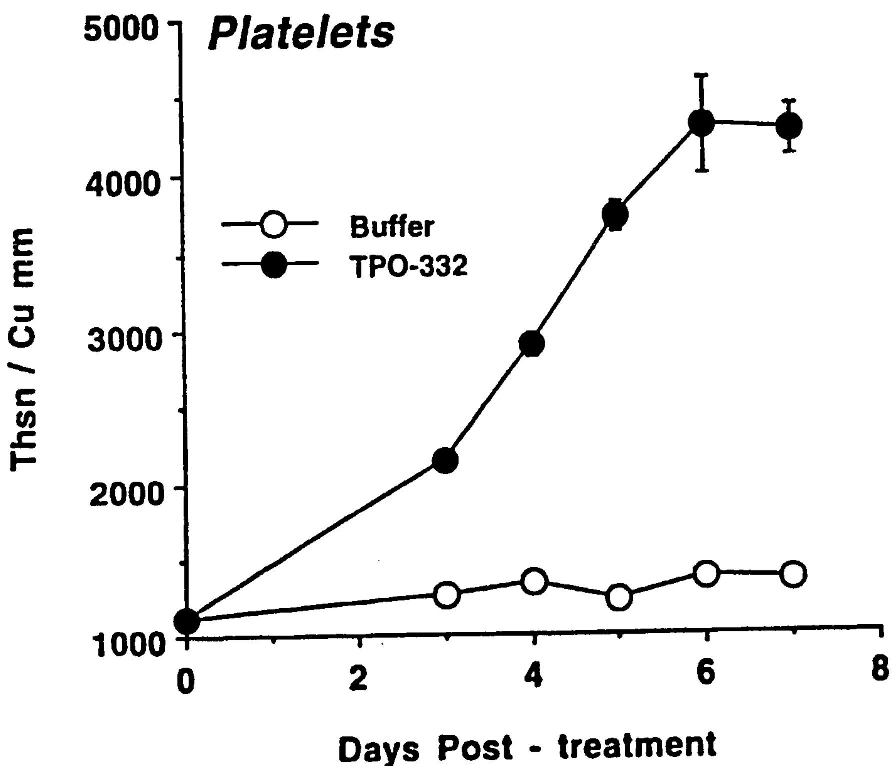


FIG.27A

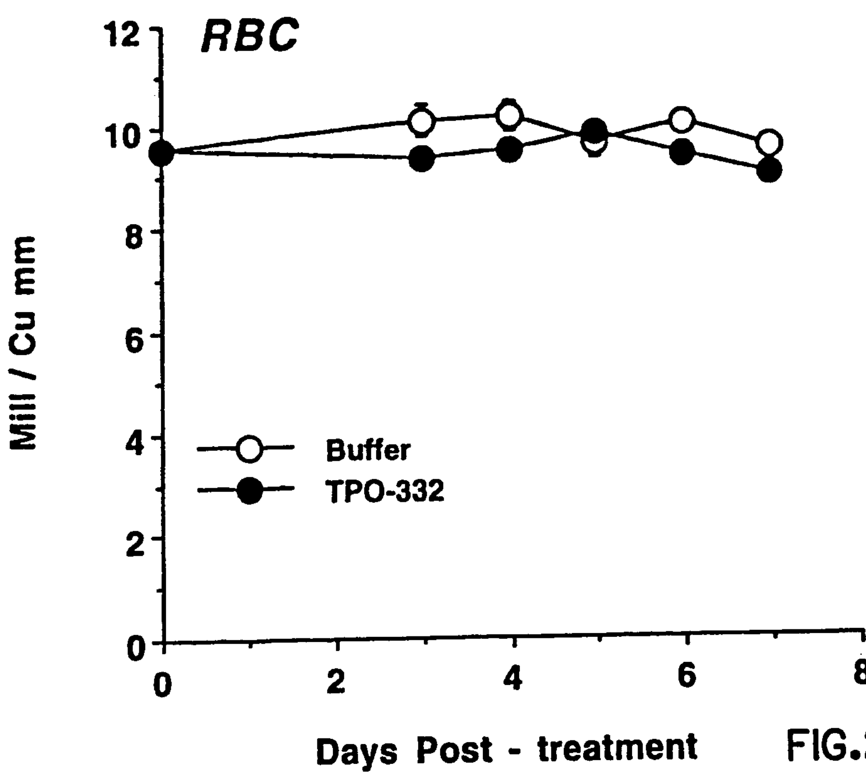


FIG.27B

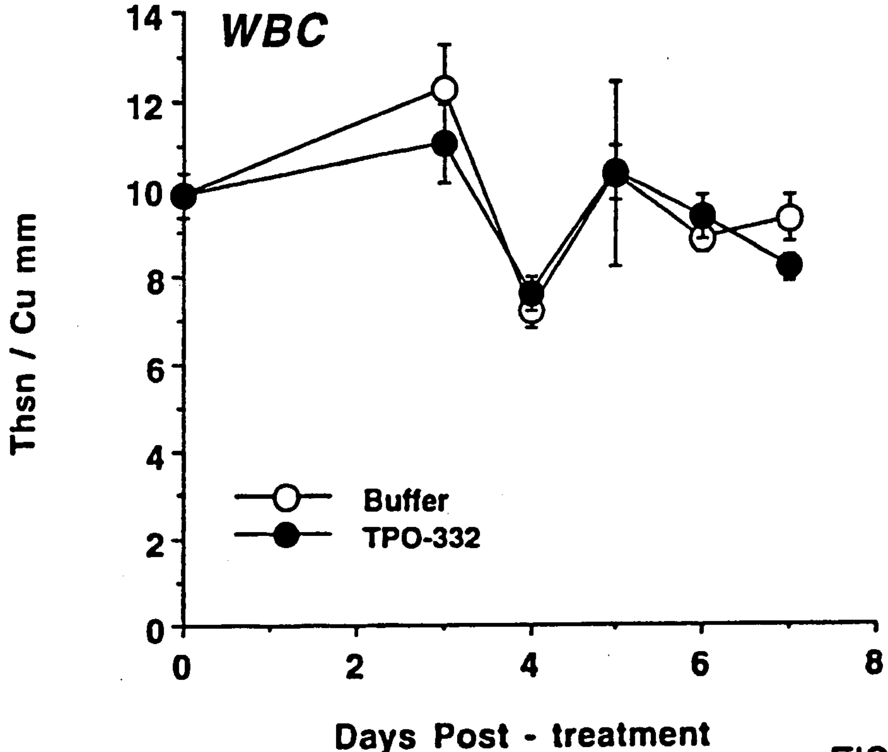


FIG.27C

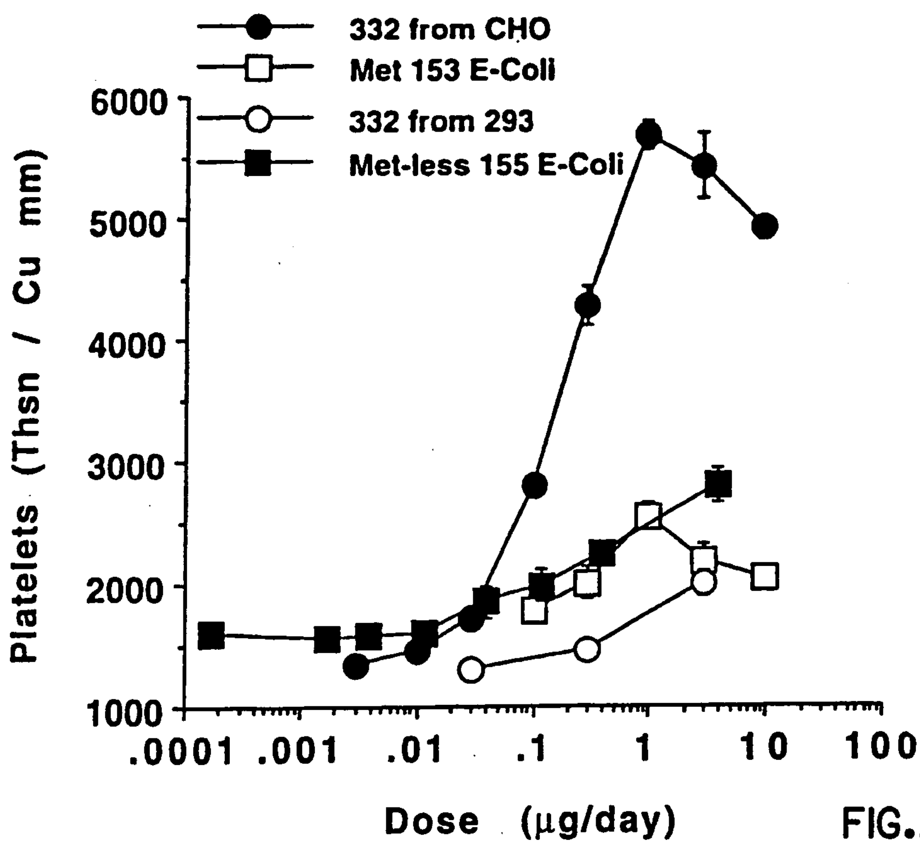


FIG.28

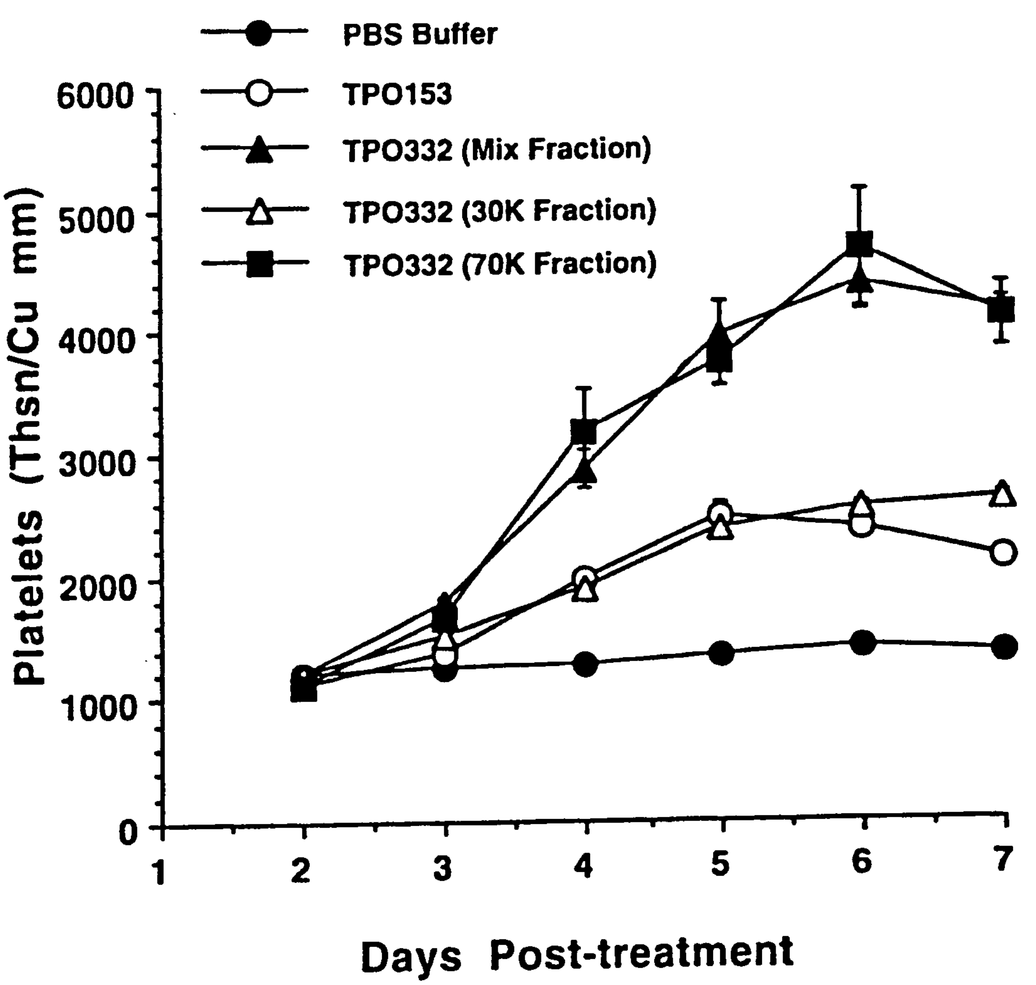

PBS Buffer
TPO153
TPO332 (Mix Fraction)
TPO332 (30K Fraction)
TPO332 (70K Fraction)
Platelets (Thsn/Cu mm)
6000
5000
4000
3000
2000
1000
0
1
2
3
4
5
6
7
Days Post-treatment

FIG.29

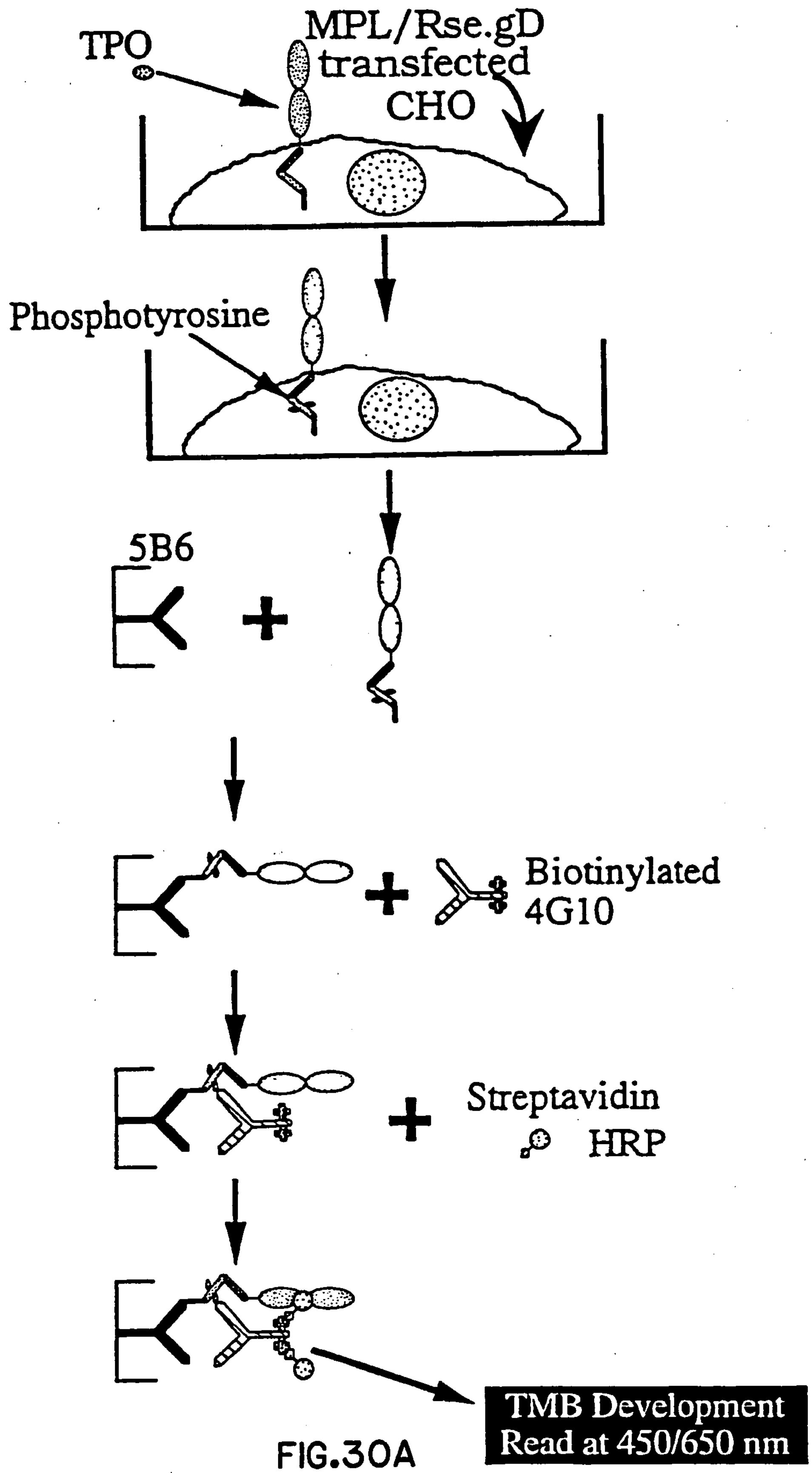
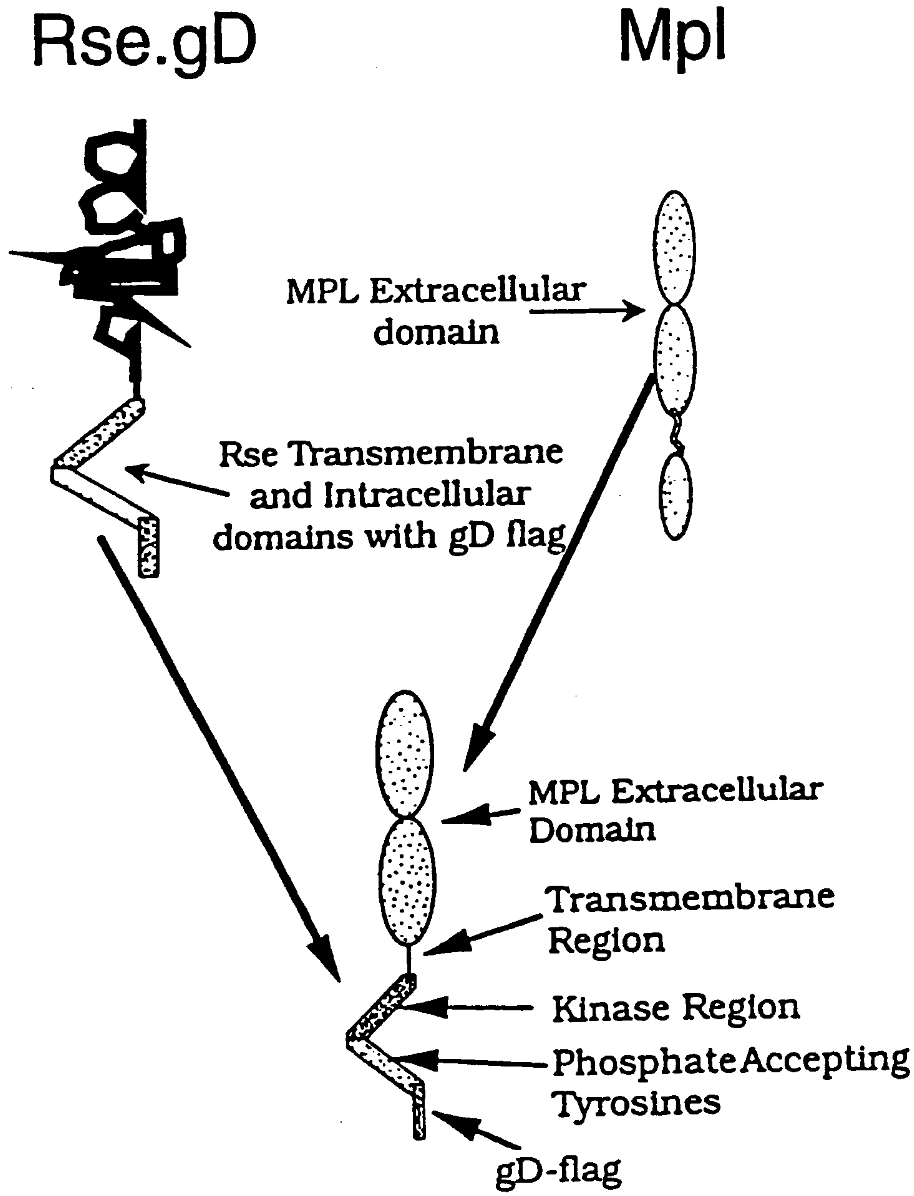

TPO
MPL/Rse.gD
transfected
CHO
Phosphotyrosine
5B6
Biotinylated
4G10
Streptavidin
HRP
TMB Development
Read at 450/650 nm

FIG.30A



MPL/Rse.gD

FIG.30B

5x10[4] MPL/Rse.gD cells
per well in 96-well
culture plates
(flat-bottom)

Stimulate w/ ligand x 30
minutes

Stimulation may be done
in either 50 or 100 µl

Solublize in:
150 mM NaCl
0.5 % Triton-X 100
50 mM HEPES, pH 7.5
Na_3VO_4 (phosphatase
inhibitor)
Leupeptin, Aprotinin
& AEBSF
(protease inhibitors)

Transfer 85 µl of cell lysate to ELISA well coated
with capture antibody, 5B6
(anti-gD peptide mAb)

Incubate on shaker x 2 hr

Wash plate and add biotinylated-4G10,
400 pg/ml, 100 µl/well.

Incubate on shaker x 2 hr

Wash plate and add Zymed streptavidin/HRP
1:10K, 100 µl/well.

Incubate on shaker x 30 min

Wash plate and add Kirkegaard & Perry TMB
substrate, 100 µl/well.

Develop x 10 min

Stop with 1 M H_3PO_4 and read at 450/650 nM

FIG.31

```
      aluI
     sstI
     sacI                                              aluI
     hgiJII                                    sau3AI pvuII
     hgiAI/aspHI                               mboI/ndeII(dam-)
     ecl136II                                  dpnI(dam+)
     bsp1286                                  pvuI/bspCI
     bsiHKAI                              pleI dpnII(dam-)
     bmyI                                 hinfI  taqI(dam-)
     banII                            rmaI    mcrI    nspBII
    taqI                              maeI   taqI(dam-)
  1 TTCGAGCTCG CCCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG GAATGTGTGT CAGTTAGGGT
    AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCTCAGCTAG CTGTCGACAC CTTACACACA GTCAATCCCA

               nlaIV
              scrFI                                 sfaNI                  scrFI
              mvaI                                 ppu10I                  mvaI
              ecoRII                               nsiI/avaIII             ecoRII
              dsaV                                nlaIII                   dsaV
              bstNI                              sphI                      bstNI
              apyI(dcm+)                         nspI                      apyI
             bsaJI                               nspHI                    sexAI
 71 GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG
    CACCTTTCAG GGGTCCGAGG GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC
```

FIG.32A

```
                           nlaIV
             scrFI                                        sfaNI
                   mvaI                                                      ppu10I
             ecoRII                                    nsiI/avaIII
             dsaV                                     nlaIII
                   bstNI                                         sphI
   [dcm+]          apyI[dcm+]                                    nspI
                  bsaJI                                          nspHI
141 GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC
    CACACCTTTC AGGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG TAGAGTTAAT CAGTCGTTGG

                                                                          nlaIII
                                                                         styI
                                                                         ncoI
                                    aciI                             bslI dsaI
          aciI          aciI  fokI              aciI bsrI  aciI      aciI bsaJI
211 ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG
    TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC

                                                 fnu4HI
                                                 bglI
                                                sfiI
                                                haeIII/palI
                                              mnlI   mnlI     ddeI
                                         haeIII/palI bsaJI  mnlI   aluI
                                       mnlI bsaJI aciI   haeIII/palI           mnlI
281 GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG
    CGACTGATTA AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC
```

FIG.32B

```
                     rmaI
                    styI
                    bsaJI
                    blnI
                    avrII                                          haeIII/palI
                   haeIII/palI                   aluI           mcrI
                  stuI                          rmaI            eagI/xmaIII/eclXI
     mnlI         haeI                          maeI            eaeI
                 mnlI maeI                     nheI             cfrI
                                               aluI             mspI
                                                                hpaII
351 AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTA GCTTATCCGG
    TCCTCCGAAA AAACCTCCGG ATCCGAAAAC GTTTTTCGAT CGAATAGGCC

                       tfiI
    scrFI              hinFI
    nciI              aciI
    mspI              thaI
    hpaII             fnuDII/mvnI                              aciI
    dsaV              bstUI                        maeII   rsaI
    cauII             bsh1236I                   maeIII    csp6I  scfI
401 CCGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTGACGTAA GTACCGCCTA TAGAGCGATA
    GGCCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCACTGCATT CATGGCGGAT ATCTCGCTAT
                                                  ^splice donor

                 fnu4HI
                 bbvI
                nspBII                                                pflMI
     mnlI       aciI       nlaIII taqI          sfaNI                 bslI
471 AGAGGATTTT ATCCCCGCTG CCATCATGGT TCGACCATTG AACTGCATCG TCGCCGTGTC CCAAAATATG
    TCTCCTAAAA TAGGGGCGAC GGTAGTACCA AGCTGGTAAC TTGACGTAGC AGCGGCACAG GGTTTTATAC
                   DHFR ATG^
```

FIG.32C

```
                                 haeIII/palI
                                haeI
                              scrFI
                              mvaI     bsrBI
                              ecoRII
                              dsaV
                              bstNI    aciI                  rsaI
                  bsmAI       apyI[dcm+]          xmnI       csp6I
                  bsaI       bsaJI  mnlI  ddeI   asp700     scaI
541 GGGATTGGCA AGAACGGAGA CCTACCCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC CAAAGAATGA
    CCCTAACCGT TCTTGCCTCT GGATGGGACC GGAGGCGAGT CCTTGCTCAA GTTCATGAAG GTTTCTTACT

                                                              scrFI
                                                              mvaI
                                                              ecoRII
         eco57I                                               dsaV
        mboII                 tfiI                            bstNI
       earI/ksp632I           hinfI                           apyI[dcm+]
      mnlI                  alwNI    hphI                    sexAI          ddeI
611 CCACAACCTC TTCAGTGGAA GGTAAACAGA ATCTGGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC
    GGTGTTGGAG AAGTCACCTT CCATTTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG

      tfiI        tru9I         tru9I
      hinfI       mseI          mseI         ddeI
   mboII taqI   ahaIII/draI    aseI/asnI/vspI                         bslI   mnlI
681 TGAGAAGAAT CGACCTTTAA AGGACAGAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCACGA
    ACTCTTCTTA GCTGGAAATT TCCTGTCTTA ATTATATCAA GAGTCATCTC TTGAGTTTCT TGGTGGTGCT
```

FIG.32D

```
    sstI
    sacI
    hgiJII
    hgiAI/aspHI
    ecl136II
    bsp1286
    bsiHKAI
    bmyI                                              tru9I                  mspI
    banII                                            aflII/bfrI              hpaII
     aluI            bstXI       fokI  sfaNI mseI                           bsaWI
751 GGAGCTCATT TTCTTGCCAA AAGTTTGGAT GATGCCTTAA GACTTATTGA ACAACCGGAA TTGGCAAGTA
    CCTCGAGTAA AAGAACGGTT TTCAAACCTA CTACGGAATT CTGAATAACT TGTTGGCCTT AACCGTTCAT

                                                                      haeIII/palI
                                                                     haeI
                                                 scrFI         scrFI
                                                 mvaI                mvaI
                                                 ecoRII        ecoRII
                                                 dsaV   tfiI   dsaV
                                                 bstNI    nlaIII     bstNI     ddeI
      accI nlaIII               mnlI             apyI[dcm+]  hinfI   apyI[dcm+]
821 AAGTAGACAT GGTTTGGATA GTCGGAGGCA GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT
    TTCATCTGTA CCAAACCTAT CAGCCTCCGT CAAGACAAAT GGTCCTTCGG TACTTAGTTG GTCCGGTGGA
```

FIG.32E

```
                          nlaIII
                      sau3AI
                      mboI/ndeII(dam-)
                      dpnI(dam+)                      maeII
     pleI             dpnII(dam-)                    aflIII
     hinfI     maeIII alwI(dam-)  apoI          maeIII
891 TAGACTCTTT GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTTT TCCCAGAAAT TGATTTGGGG
    ATCTGAGAAA CACTGTTCCT AGTACGTCCT TAAACTTTCA CTGTGCAAAA AGGGTCTTTA ACTAAACCCC

                                hgaI
                               hinlI/acyI
                               ahaII/bsaHI
                             scrFI
                             mvaI     mnlI
                             ecoRII
                             dsaV
                             bstNI    ecoNI
                             apyI(dcm+)      mnlI
            mnlI            bsaJI     bslI ddeI
961 AAATATAAAC CTCTCCCAGA ATACCCAGGC GTCCTCTCTG
    TTTATATTTG GAGAGGGTCT TATGGGTCCG CAGGAGAGAC
```

FIG.32F

```
          scrFI
          mvaI
          ecoRII
          dsaV
          bstNI
          apyI[dcm+]
       sau96I
       avaII
       asuI   mnlI        sfaNI                  accI      mboII               sfaNI
                                                                                  mboII
1001  AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAAG AAAGACTAAC AGGAAGATGC
      TCCAGGTCCT CCTTTTTCCG TAGTTCATAT TCAAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTACG
                                                                     ^END DHFR

                                                           nlaIII
                                                          styI
                                                          ncoI
                                          ppu10I          dsaI
                        mnlI      aluI    nsiI/avaIII     bsaJI
1071  TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT ATGCATTTTT ATAAGACCAT GGGACTTTTG
      AAAGTTCAAG AGACGAGGGG AGGATTTCGA TACGTAAAAA TATTCTGGTA CCCTGAAAAC
```

FIG.32G

```
                  styI
                  bsaJI
              sau3AI                      fnu4HI
              mboI/ndeII[dam-]            aciI
              dpnI[dam+]                 thaI
              dpnII[dam-]                fnuDII/mvnI tru9I
              alwI[dam-]                 bstUI       mseI
             bstYI/xhoII                 bsh1236I   aseI/asnI/vspI
1131 CTGGCTTTAG ATCCCCTTGG CTTCGTTAGA ACGCGGCTAC AATTAATACA TAACCTTATG TATCATACAC
     GACCGAAATC TAGGGGAACC GAAGCAATCT TGCGCCGATG TTAATTATGT ATTGGAATAC ATAGTATGTG

                                                                       sau96I
                                                                       avaII
                                                                       asuI
                                                                     scrFI
                                                                     mvaI
                                                                     ecoRII
                                                                     dsaV
                                                                     bstNI
                 maeIII                                              apyI[dcm+]
                hphI    scfI        fokI                        bslI  bsaJI
1201 ATACGATTTA GGTGACACTA TAGATAACAT CCACTTTGCC TTTCTCTCCA CAGGTGTCCA CTCCCAGGTC
     TATGCTAAAT CCACTGTGAT ATCTATTGTA GGTGAAACGG AAAGAGAGGT GTCCACAGGT GAGGGTCCAG
```

FIG.32H

```
                                                                  scrFI
                                                                  nciI
                                                                  mspI
                                                                  hpaII
                                                                  dsaV
                                                                 xmaI/pspAI
                                                                 smaI
                                                                 scrFI
                                                                 nciI
                                                                 dsaV
                                                                 cauII
                                                                 bsaJI
                                                                 avaI
                                                             sau3AI
                                                             mboI/ndeII[dam-]
                                                             dpnI[dam+]
                                                             dpnII[dam-]
                                               pleI         nlaIV cauII
                                               hinfI        bstYI/xhoII
                         scfI                taqI   rmaI    bamHI bsaJI
                         aluI  pstI         salI   maeI    alwI[dam-]
            mnlI        hindIII  bspMI     hincII/hindII alwI[dam-]
            bsaJI     ddeI     bsgI        accI  xbaI mnlI  bsaJI
1271 CAACTGCACC TCGGTTCTAA GCTTCTGCAG GTCGACTCTA GAGGATCCCC
     GTTGACGTGG AGCCAAGATT CGAAGACGTC CAGCTGAGAT CTCCTAGGGG
```

FIG.32I

```
                        sau96I
                aciI    haeIII/palI
               fnu4HI   asuI
               bglI  nlaIII
              sfiI  styI
             eaeI   ncoI
             cfrI   dsaI                            aluI
   ecoRI    taqI haeIII/palI                       fnu4HI
   apoI    claI/bsp106 bsaJI                      bbvI        maeIII
1321 GGGGAATTCA ATCGATGGCC GCCATGGCCC AACTTGTTTA TTGCAGCTTA TAATGGTTAC AAATAAAGCA
     CCCCTTAAGT TAGCTACCGG CGGTACCGGG TTGAACAAAT AACGTCGAAT ATTACCAATG TTTATTTCGT
                                      ^sv40 early poly A

                                                    rmaI
   sfaNI    apoI                               bsmI maeI
1391 ATAGCATCAC AAATTTCACA AATAAAGCAT TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT
     TATCGTAGTG TTTAAAGTGT TTATTTCGTA AAAAAAGTGA CGTAAGATCA ACACCAAACA GGTTTGAGTA
```

FIG.32J

```
                          sau3AI
                          mboI/ndeII(dam-)
                          dpnI(dam+)
                          dpnII(dam-)
                         pvuI/bspCI
                         mcrI
                        taqI(dam-)  tru9I
                       claI/bsp106(dam-)
                      sau3AI       mseI
                      mboI/ndeII(dam-)
                      dpnI(dam+) xmnI
                      dpnII(dam-)  aseI/asnI/vspI
             nlaIII  alwI(dam-)  asp700
1461 CAATGTATCT TATCATGTCT GGATCGATCG GGAATTAATT
     GTTACATAGA ATAGTACAGA CCTAGCTAGC CCTTAATTAA
                      sv40 origin^

                                                           rsaI
              haeIII/palI                                  csp6I
             haeI                                         nlaIV
          styI                                            kpnI
     fnu4HI ncoI                                          hgiCI
     bbvI   dsaI                                          banI
   hinPI    bsaJI                                         asp718     mnlI
   hhaI/cfoI nlaIII          mnlI       mnlI              acc65I   ddeI  aciI
1501 CGGCGCAGCA CCATGGCCTG AAATAACCTC TGAAAGAGGA ACTTGGTTAG GTACCTTCTG AGGCGGAAAG
     GCCGCGTCGT GGTACCGGAC TTTATTGGAG ACTTTCTCCT TGAACCAATC CATGGAAGAC TCCGCCTTTC
```

FIG.32K

FIG.32L

```
                                                         nlaIV
                                                      scrFI
                                                      mvaI
                                                      ecoRII
                                                      dsaV
        aluI                                          bstNI
       pvuII                                          apyI[dcm+]
       nspBII                                        bsaJI
1571 AACCAGCTGT GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC
     TTGGTCGACA CCTTACACAC AGTCAATCCC ACACCTTTCA GGGGTCCGAG GGGTCGTCCG TCTTCATACG

                                                           nlaIV
      sfaNI                scrFI          scrFI
          ppu10I                 mvaI                     mvaI
    nsiI/avaIII            ecoRII         ecoRII
   nlaIII                  dsaV           dsaV
        sphI                     bstNI                    bstNI
        nspI                     apyI[dcm+]               apyI[dcm+]
        nspHI                   sexAI                    bsaJI
1641 AAAGCATGCA TCTCAATTAG TCAGCAACCA GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT
     TTTCGTACGT AGAGTTAATC AGTCGTTGGT CCACACCTTT CAGGGGTCCG AGGGGTCGTC CGTCTTCATA

              sfaNI
            ppu10I
            nsiI/avaIII
           nlaIII
          sphI
          nspI                                                         aciI
          nspHI                                 aciI         aciI  fokI
1711 GCAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCG CCCCTAACTC CGCCCATCCC GCCCCTAACT
     CGTTTCGTAC GTAGAGTTAA TCAGTCGTTG GTATCAGGGC GGGGATTGAG GCGGGTAGGG CGGGGATTGA
```

```
                              nlaIII
                             styI
                             ncoI
       bsrI              bslI dsaI
     asiI        aciI    aciI bsaJI                                mnlI
1781 CCGCCCAGTT CCGCCCATTC TCCGCCCCAT GGCTGACTAA TTTTTTTTAT TTATGCAGAG
     GGCGGGTCAA GGCGGGTAAG AGGCGGGGTA CCGACTGATT AAAAAAAATA AATACGTCTC

                                                                   styI
        fnu4HI                                                     bsaJI
        bglI                                                       blnI
       sfiI                                                        avrII
       haeIII/palI                                               haeIII/palI
     mnlI    mnlI     ddeI                                       stuI
  haeIII/palI bsaJI mnlI  aluI                          mnlI     haeI
    bsaJI aciI    haeIII/palI                        mnlI       mnlI
1841 GCCGAGGCCG CCTCGGCCTC TGAGCTATTC CAGAAGTAGT GAGGAGGCTT TTTTGGAGGC
     CGGCTCCGGC GGAGCCGGAG ACTCGATAAG GTCTTCATCA CTCCTCCGAA AAAACCTCCG
```

FIG.32M

```
                                                        aciI             hinPI
                                                      haeIII/palI       hhaI/cfoI
                                                     mcrI              thaI
                                                     eagI/xmaIII/eclXI fnuDII/mvnI
                                              taqI   eaeI              bstUI               bspMI
                                             xhoI   notI              hinPI               scfI
                                             paeR7I cfrI    tru9I     hhaI/cfoI tru9I     pstI
                                             avaI   fnu4HI  pacI     ascI      ahaIII/draI
     rmaI                                   mnlI   aciI    mseI tru9I bsh1236I mseI      bsgI
     maeI                        aluI maeIII    bsrBI fnu4HI    mseI bssHII   swaI      sse8387I
1901 CTAGGCTTTT GCAAAAAGCT GTTACCTCGA GCGGCCGCTT AATTAAGGCG CGCCATTTAA ATCCTGCAGG
     GATCCGAAAA CGTTTTTCGA CAATGGAGCT CGCCGGCGAA TTAATTCCGC GCGGTAAATT TAGGACGTCC
                          ^start pUC118
                                  ^linearization linker inserted into Hpa1 site

                                                                 scrFI
                                                                 mvaI
                                                                 ecoRII
                           haeIII/palI                           dsaV
                          eaeI                                   bstNI
   maeIII                 cfrI                   maeIII          apyI[dcm+]         tru9I
         aluI            bsrI               maeII    bsrI      bsaJI    maeIII     mseI
1971 TAACAGCTTG GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT
     ATTGTCGAAC CGTGACCGGC AGCAAAATGT TGCAGCACTG ACCCTTTTGG GACCGCAATG GGTTGAATTA
```

FIG.32N

```
                                                            sau3AI
                                                       sau96I     mboI/ndeII[dam-]
                                                 haeIII/palI
                                                 asuI         dpnI[dam+]
                        aluI                        mnlI           dpnII[dam-]
     fnu4HI            pvuII                     mboII     aciI  pvuI/bspCI
     bbvI    fokI      nspBII                    earI/ksp632I    mcrI
2041 CGCCTTGCAG CACATCCCCC CTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT
     GCGGAACGTC GTGTAGGGGG GAAGCGGTCG ACCGCATTAT CGCTTCTCCG GGCGTGGCTA

                                            hinPI
                                            hhaI/cfoI
                                           nlaIV
                                           narI
                                           kasI
                                           hinlI/acyI
                                           hgiCI
                                           haeII     aciI
                                           banI   sfaNI
                       bglI                ahaII/bsaHI                  sfaNI
2101 CGCCCTTCCC AACAGTTGCG TAGCCTGAAT GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC
     GCGGGAAGGG TTGTCAACGC ATCGGACTTA CCGCTTACCG CGGACTACGC CATAAAAGAG GAATGCGTAG
```

FIG.32O

```
                                                hinPI
                                               thaI
                                               fnuDII/mvnI
                                               bstUI scfI     hinPI
                                               bsh1236I        hhaI/cfoI
                                           rsaI hhaI/cfoI  fnu4HI
     aciI          aciI   maeII            csp6I   bslI    aciI
2171 TGTGCGGTAT TTCACACCGC ATACGTCAAA GCAACCATAG TACGCGCCCT GTAGCGGCGC
     ACACGCCATA AAGTGTGGCG TATGCAGTTT CGTTGGTATC ATGCGCGGGA CATCGCCGCG

         fnu4HI                fnu4HI
        thaI                 hinPI
        fnuDII/mvnI          hhaI/cfoI                              hinPI
        bctUI               thaI                                    hhaI/cfoI
       hinPI aciI           fnuDII/mvnI                         rmaI
       hhaI/cfoI            bstUI                           hinPI   haeII
   tru9I aciI               bsh1236I      aciI              hhaI/cfoI   bsrBI
   mseI bsh1236I         maeIII bbvI  maeIII               haeII maeI    aciI
2231 ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT
     TAATTCGCGC CGCCCACACC ACCAATGCGC GTCGCACTGG CGATGTGAAC GGTCGCGGGA TCGCGGGCGA

                                                                      nlaIV
                                          mspI                       hgiJII
                                          hpaII                      bsp1286
                                         naeI                        bmyI
               mboII                 maeII cfr10I         aluI       banII
2301 CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG TCAAGCTCTA AATCGGGGGC
     GGAAAGCGAA AGAAGGGAAG GAAAGAGCGG TGCAAGCGGC CGAAAGGGGC AGTTCGAGAT TTAGCCCCCG
```

FIG.32P

```
                                                         mnlI
                                                    nlaIV
                                                    hgiCI
                 nlaIV                              banI  taqI                           hphI
2371 TCCCTTTAGG GTTCCGATTT AGTGCTTTAC GGCACCTCGA CCCCAAAAAA CTTGATTTGG
     AGGGAAATCC CAAGGCTAAA TCACGAAATG CCGTGGAGCT GGGGTTTTTT GAACTAAACC

                   maeII    haeIII/palI
                 draIII    sau96I                                       maeII pleI
                 bsaAI     asuI                                        drdI   hinfI  maeII
2401 GTGATGGTTC ACGTAGTGGG CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT
     CACTACCAAG TGCATCACCC GGTAGCGGGA CTATCTGCCA AAAAGCGGGA AACTGCAACC TCAGGTGCAA

       tru9I      pleI                                   bslI
       mseI       hinfI          bsrI                   bslI  avaI
2501 CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT CGGGCTATTC TTTTGATTTA
     GAAATTATCA CCTGAGAACA AGGTTTGACC TTGTTGTGAG TTGGGATAGA GCCCGATAAG AAAACTAAAT

                                                                                tru9I
                                                 tru9I              tru9I       mseI
                                    haeIII/palI mseI        aluI    mseI    apoI
2571 TAAGGGATTT TGCCGATTTC GGCCTATTGG  TTAAAAAATG AGCTGATTTA ACAAAAATTT
     ATTCCCTAAA ACGGCTAAAG CCGGATAACC  AATTTTTTAC TCGACTAAAT TGTTTTTAAA
```

FIG.32Q

```
                                                                  hgiAI/aspHI
     thaI                                                         bsp1286
     fnuDII/mvnI                     maeII                        bsiHKAI
        apoI                       psp1406I                       bmyI   ddeI
     bstUI    tru9I              tru9I                            apaLI/snoI  rsaI
     bsh1236I mseI    sspI mseI                                   alw44I/snoI csp6I
2631 AACGCGAATT TTAACAAAAT ATTAACGTTT ACAATTTTAT GGTGCACTCT CAGTACAATC
     TTGCGCTTAA AATTGTTTTA TAATTGCAAA TGTTAAAATA CCACGTGAGA GTCATGTTAG

                                                                       bsrI           hinPI
                                                                    maeIII          fnu4HI
              fnu4HI      tru9I                                   maeII         nlaIII hhaI/cfoI
           sfaNI aciI     mseI            aciI                  bsaAI tth111I/aspI bbvI
2691 TGCTCTGATG  CCGCATAGTT AAGCCAACTC CGCTATCGCT ACGTGACTGG GTCATGGCTG CGCCCCGACA
     ACGAGACTAC  GGCGTATCAA TTCGGTTGAG GCGATAGCGA TGCACTGACC CAGTACCGAC GCGGGGCTGT

                             hinPI
                             hhaI/cfoI                             sfaNI
                            thaI                                mspI
                            fnuDII/mvnI                         hpaII
                            bstUI                              scrFI
                            nspBII bsh1236I                    nciI
                                                               dsaV fokI                maeIII
       aciI          aciI hgaI        drdI                     cauII     aciI       aluI
2761 CCCGCCAACA CCCGCTGACG CGCCCTGACG GGCTTGTCTG CTCCCGGCAT CCGCTTACAG ACAAGCTGTG
     GGGCGGTTGT GGGCGACTGC GCGGGACTGC CCGAACAGAC GAGGGCCGTA GGCGAATGTC TGTTCGACAC
```

FIG.32R

```
                                                                         thaI
                                                                          fnuDII/mvnI
           scrFI                                                          bstUI
           nciI                                                           bsh1236I
           mspI                                                          hinPI
           hpaII      nspI                                               hhaI/cfoI
           dsaV       nspHI                                             thaI mnlI
      esp3I        fnu4HI                                               fnuDII/mvnI
       bsmAI       bbvI                                                 bstUI
     bslI   cauII aluI nlaIII    mnlI     hphI       hphI       bsh1236I
2831 ACCGTCTCCG GGAGCTGCAT GTGTCAGAGG TTTTCACCGT CATCACCGAA ACGCGCGAGG CAGTATTCTT
     TGGCAGAGGC CCTCGACGTA CACAGTCTCC AAAAGTGGCA GTAGTGGCTT TGCGCGCTCC GTCATAAGAA

                   mnlI
                 haeIII/palI
     mboII      sau96I                                      nlaIII
     bpuAI      asuI                                 tru9I  rcaI
     bbsI     eco0109I/draII                         mseI   bspHI
2901 GAAGACGAAA GGGCCTCGTG ATACGCCTAT TTTTATAGGT TAATGTCATG
     CTTCTGCTTT CCCGGAGCAC TATGCGGATA AAAATATCCA ATTACAGTAC

                                                             aciI
                                                            thaI
                                                            fnuDII/mvnI
                       hinII/acyI                           bstUI
                       ahaII/bsaHI                          bsh1236I
                       aatII                               hinPI
                   ddeI maeII                              hhaI/cfoI
2951 ATAATAATGG TTTCTTAGAC GTCAGGTGGC ACTTTTCGGG GAAATGTGCG
     TATTATTACC AAAGAATCTG CAGTCCACCG TGAAAAGCCC CTTTACACGC
```

FIG.32S

```
                                                                              bsmAI
                                                                         rcaI
                                                                   bsrBI nlaIII
      nlaIV                                                        aciI bspHI
3001 CGGAACCCCT ATTTGTTTAT TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA
     GCCTTGGGGA TAAACAAATA AAAAGATTTA TGTAAGTTTA TACATAGGCG AGTACTCTGT TATTGGGACT

                                   mboII
                    sspI           earI/ksp632I
3071 TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT CCGTGTCGCC CTTATTCCCT
     ATTTACGAAG TTATTATAAC TTTTTCCTTC TCATACTCAT AAGTTGTAAA GGCACAGCGG GAATAAGGGA

        fnu4HI
        aciI                                hphI            hphI            sfaNI
3141 TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA AACGCTGGTG AAAGTAAAAG
     AAAAACGCCG TAAAACGGAA GGACAAAAAC GAGTGGGTCT TTGCGACCAC TTTCATTTTC

                         hgiAI/aspHI
                         bsp1286                                sau3AI
                sau3AI   bsiHKAI                                mboI/ndeII[dam-]
                mboI/ndeII[dam-]                                dpnI[dam+]
                dpnI[dam+] bmyI                                 dpnII[dam-]
                dpnII[dam-]                                    bstYI/xhoII
             mboII[dam-]   apaLI/snoI            taqI          alwI[dam-]  aciI
           eco57I          alw44I/snoI       maeIII       bsrI           nspBII
3201 ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA ACTGGATCTC AACAGCGGTA
     TACGACTTCT AGTCAACCCA CGTGCTCACC CAATGTAGCT TGACCTAGAG TTGTCGCCAT
```

FIG.32T

```
        sau3AI
        mboI/ndeII[dam-]               maeII
        dpnI[dam+]                    psp1406I          hgiAI/aspHI
        dpnII[dam-]                  xmnI               bsp1286  tru9I
        alwI[dam-]                   asp700             bsiHKAI  mseI
       bstYI/xhoII                 mboII                bmyI    ahaIII/draI
3261   AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT GATGAGCACT TTTAAAGTTC
       TCTAGGAACT CTCAAAAGCG GGGCTTCTTG CAAAAGGTTA CTACTCGTGA AAATTTCAAG

                                          scrFI
                 aciI                     nciI
                thaI                      mspI
                fnuDII/mvnI               hpaII
                bstUI                     dsaV
                bsh1236I               hinlI/acyI
              hinPI                    hgaI cauII                   aciI
              hhaI/cfoI                ahaII/bsaHI     bcgI  mcrI  fnu4HI
3321   TGCTATGTGG CGCGGTATTA TCCCGTGATG ACGCCGGGCA AGAGCAACTC GGTCGCCGCA
       ACGATACACC GCGCCATAAT AGGGCACTAC TGCGGCCCGT TCTCGTTGAG CCAGCGGCGT

                                      rsaI
                                      csp6I   bsrI
              ddeI                   scaI  hphI  maeIII      sfaNI    fokI
3381   TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT CACAGAAAAG CATCTTACGG
       ATGTGATAAG AGTCTTACTG AACCAACTCA TGAGTGGTCA GTGTCTTTTC GTAGAATGCC
```

FIG.32U

```
                                                                           haeIII/palI
                                                                          eaeI
                                                                          cfrI
                                  fnu4HI                                 fnu4HI
     nlaIII                       bbvI           nlaIII                  aciI
3441 ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC CATGAGTGAT AACACTGCGG CCAACTTACT
     TACCGTACTG TCATTCTCTT AATACGTCAC GACGGTATTG GTACTCACTA TTGTGACGCC GGTTGAATGA

               sau96I
               avaII
          sau3AI   asuI                                                 nlaIII
          mboI/ndeII[dam-]                                           sau3AI maeIII
          dpnI[dam+]                                                 mboI/ndeII[dam-]
          dpnII[dam-]                                                dpnI[dam+]
         pvuI/bspCI                                                  dpnII[dam-]
         mcrI   mnlI          aluI    aciI                     nlaIII alwI[dam-]
3511 TCTGACAACG ATCGGAGGAC CGAAGGAGCT AACCGCTTTT TTGCACAACA TGGGGGATCA TGTAACTCGC
     AGACTGTTGC TAGCCTCCTG GCTTCCTCGA TTGGCGAAAA AACGTGTTGT ACCCCCTAGT ACATTGAGCG

              mspI
    sau3AI   nlaIV
    mboI/ndeII[dam-] aluI
    dpnI[dam+]    hpaII                                                        fnu4HI
    dpnII[dam-] bsaWI                                    maeIII     sfaNI    bbvI
3581 CTTGATCGTT GGGAACCGGA GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCAGCAG
     GAACTAGCAA CCCTTGGCCT CGACTTACTT CGGTATGGTT TGCTGCTCGC ACTGTGGTGC TACGGTCGTC
```

FIG.32V

```
                       hinPI                                                   mspI
                       hhaI/cfoI                                               hpaII
                      mstI                                                    scrFI
                      aviII/fspI       bsrI                              aluI  nciI
                   maeII              tru9I                             rmaI   dsaV
                  psp1406I            mseI                              maeI   cauII
3651 CAATGGCAAC AACGTTGCGC AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC
     GTTACCGTTG TTGCAACGCG TTTGATAATT GACCGCTTGA TGAATGAGAT CGAAGGGCCG

                                                                        bglI
                                                                       sau96I
       tru9I       fokI                          sau96I                haeIII/palI
       mseI     bsrI         aciI                avaII       hinPI     asuI     mspI
      aseI/asnI/vspI       mnlI                  asuI        hhaI/cfoI          hpaII
3711 AACAATTAAT AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG
     TTGTTAATTA TCTGACCTAC CTCCGCCTAT TTCAACGTCC TGGTGAAGAC GCGAGCCGGG AAGGCCGACC

                            mspI                     thaI
                            hpaII                    fnuDII/mvnI
                           cfr10I                    bstUI
                         nlaIV hphI              bsmAI aciI          fnu4HI
                       gsuI/bpmI                bsaI  bsh1236I       bbvI
3781 CTGGTTTATT GCTGATAAAT CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC
     GACCAAATAA CGACTATTTA GACCTCGGCC ACTCGCACCC AGAGCGCCAT AGTAACGTCG
```

FIG.32W

```
       sau96I
       asuI
      nlaIV                                                        pleI
    bsrI haeIII/palI      mnlI                                      hinfI
                                                            eam1105I
3841 ACTGGGGCCA GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC
     TGACCCCGGT CTACCATTCG GGAGGGCATA GCATCAATAG ATGTGCTGCC CCTCAGTCCG

                               ddeI
                            sau3AI          nlaIV
                            mboI/ndeII[dam-]
                            dpnI[dam+]      hgiCI          tru9I
          fokI              dpnII[dam-]     banI mnlI      mseI
3901 AACTATGGAT GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
     TTGATACCTA CTTGCTTTAT CTGTCTAGCG ACTCTATCCA CGGAGTGACT AATTCGTAAC

                                                  tru9I
                                                  mseI          tru9I
     maeIII                                      ahaIII/draI    mseI
3961 GTAACTGTCA GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA
     CATTGACAGT CTGGTTCAAA TGAGTATATA TGAAATCTAA CTAAATTTTG AAGTAAAAAT

               rmaI       sau3AI
           sau3AI hphI    mboI/ndeII[dam-]
           mboI/ndeII[dam-]
           dpnI[dam+]     dpnI[dam+]
           dpnII[dam-]    dpnII[dam-]
       tru9I bstYI/xhoII  alwI[dam-]          nlaIII             maeII
      mseI  alwI[dam-]   bstYI/xhoII         rcaI            tru9I
     ahaIII/draI maeI  mboII[dam-]           bspHI           mseI
4021 ATTTAAAAGG ATCTAGGTGA AGATCCTTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTTCG
     TAAATTTTCC TAGATCCACT TCTAGGAAAA ACTATTAGAG TACTGGTTTT AGGGAATTGC ACTCAAAAGC
```

FIG.32X

FIG.32Y

```
                                                          sau3AI
                                                          mboI/ndeII[dam-]
                                                          dpnI[dam+]   sau3AI
                                                          dpnII[dam-]  mboI/ndeII[dam-]
                                                         bstYI/xhoII   dpnI[dam+]
                                                sau3AI   alwI[dam-]    dpnII[dam-]
                                                mboI/ndeII[dam-]       alwI[dam-]
                   hgaI                         dpnI[dam+] mboII[dam-]
               ddeI                             dpnII[dam-]            bstYI/xhoII
4091 TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTTTT
     AAGGTGACTC GCAGTCTGGG GCATCTTTTC TAGTTTCCTA GAAGAACTCT AGGAAAAAAA

        thaI
        fnuDII/mvnI
        bstUI
        bsh1236I
       hinPI        fnu4HI                                  aciI
       hhaI/cfoI    bbvI                         aciI     nspBII
4151 CTGCGCGTAA TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG
     GACGCGCATT AGACGACGAA CGTTTGTTTT TTTGGTGGCG ATGGTCGCCA CCAAACAAAC

        sau3AI
        mboI/ndeII[dam-]
        dpnI[dam+]
        dpnII[dam-]
       alwI[dam-]
     mspI                                        bsrI                  hinPI
     hpaII       aluI                         maeIII     eco57I        hhaI/cfoI
4211 CCGGATCAAG AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA CCAAATACTG
     GGCCTAGTTC TCGATGGTTG AGAAAAAGGC TTCCATTGAC CGAAGTCGTC TCGCGTCTAT GGTTTATGAC
```

```
        rmaI                 haeIII/palI
        maeI       bslI     haeI                    scfI      aciI         mnlI
4281 TCCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT
     AGGAAGATCA CATCGGCATC AATCCGGTGG TGAAGTTCTT GAGACATCGT GGCGGATGTA TGGAGCGAGA

                                                          scrFI
                                                          nciI
                         fnu4HI                           mspI
                 alwNI   bbvI                             hpaII
                bsrI  fnu4HI                              dsaV    pleI
           maeIII     bbvI     bsrI                       cauII   hinfI
4351 GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA
     CGATTAGGAC AATGGTCACC GACGACGGTC ACCGCTATTC AGCACAGAAT GGCCCAACCT GAGTTCTGCT

                                                     hgiAI/aspHI
                      nspBII                         bsp1286
         mspI        fnu4HI                          bsiHKAI
         hpaII       bbvI mcrI                       bmyI
        bsaWI       hinPI aciI                       apaLI/snoI
      maeIII        hhaI/cfoI                        alw44I/snoI  aluI
4421 TAGTTACCGG ATAAGGCGCA GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA
     ATCAATGGCC TATTCCGCGT CGCCAGCCCG ACTTGCCCCC CAAGCACGTG TGTCGGGTCG AACCTCGCTT

                                                      hinPI
                                                      hhaI/cfoI
                ddeI      scfI                       haeII
4491 CGACCTACAC CGAACTGAGA TACCTACAGC GTGAGCATTG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA
     GCTGGATGTG GCTTGACTCT ATGGATGTCG CACTCGTAAC TCTTTCGCGG TGCGAAGGGC TTCCCTCTTT
```

FIG.32Z-1

```
                                                                                 scrFI
                                                                                 mvaI
                                                                                 ecoRII     mvaI
                  mspI                                                           dsaV       ecoRII
                  hpaII                                                          bstNI
                  bslI      fnu4HI                          hinPI mnlI           bsaJI
     aciI        bsaWI      aciI                            hhaI/cfoI     aluI   apyI[dcm+]
4561 GGCGGACAGG TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC
     CCGCCTGTCC ATAGGCCATT CGCCGTCCCA GCCTTGTCCT CTCGCGTGCT CCCTCGAAGG TCCCCCTTTG

     scrFI

     dsaV
     bstNI                                                      taqI
     apyI[dcm+]                            mnlI  drdI    hgaI              sfaNI
4631 GCCTGGTATC TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTG TGATGCTCGT
     CGGACCATAG AAATATCAGG ACAGCCCAAA GCGGTGGAGA CTGAACTCGC AGCTAAAAAC ACTACGAGCA

                                             haeIII/palI
                                            fnu4HI
                                            aciI
                                           thaI bslI
                                           fnuDII/mvnI
             nlaIV                         bstUI
           aciI                            bsh1236I
4701 CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC
     GTCCCCCCGC CTCGGATACC TTTTTGCGGT CGTTGCGCCG
```

FIG.32Z-2

```
                    haeIII/palI
                  scrFI
                  mvaI bslI
                  ecoRII
                  dsaV                        nlaIII
                  bstNI                          nspI
                  apyI[dcm+]      haeIII/palI  nspHI                        tfiI
               nlaIV  haeI       haeI          aflIII                       hinfI
4741 CTTTTTACGG TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT
     GAAAAATGCC AAGGACCGGA AAACGACCGG AAAACGAGTG TACAAGAAAG GACGCAATAG GGGACTAAGA

                                                         fnu4HI
                                                        bbvI
                                                  bsrBI   aciI
                  aciI           aluI             aciI  fnu4HI       mcrI
4811 GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG CCGAACGACC
     CACCTATTGG CATAATGGCG GAAACTCACT CGACTATGGC GAGCGGCGTC GGCTTGCTGG

                                                 hinPI
        fnu4HI                                  haeII
        bbvI pleI                           sapI hhaI/cfoI
      hinPI  hinfI                          mboII                        mnlI
      hhaI/cfoI                 mnlI  aciI  earI/ksp632I              aciI
4871 GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG  GAAGAGCGCC CAATACGCAA ACCGCCTCTC
     CTCGCGTCGC TCAGTCACTC GCTCCTTCGC  CTTCTCGCGG GTTATGCGTT TGGCGGAGAG
```

FIG.32Z-3

```
       thaI
       fnuDII/mvnI
       bstUI
       bsh1236I
      hinPI
      hhaI/cfoI
     thaI
     fnuDII/mvnI
     bstUI                  tru9I  aluI
     bsh1236I haeIII/palI       pvuII
    bslI    eaeI  tfiI aseI/asnI/vspI
    aciI    cfrI  hinfI  mseI  nspBII                       bsrI     aciI
4931 CCCGCGCGTT GGCCGATTCA TTAATCCAGC TGGCACGACA GGTTTCCCGA CTGGAAAGCG
     GGGCGCGCAA CCGGCTAAGT AATTAGGTCG ACCGTGCTGT CCAAAGGGCT GACCTTTCGC

                                                               scrFI
                                                               mvaI
                                                               ecoRII
                                                               dsaV
                           tru9I                        nlaIV  bstNI
            hinPI          mseI      maeIII             hgiCI  apyI[dcm+]
            hhaI/cfoI aseI/asnI/vspI  mnlI              banI bsaJI
4991 GGCAGTGAGC GCAACGCAAT TAATGTGAGT TACCTCACTC ATTAGGCACC CCAGGCTTTA CACTTTATGC
     CCGTCACTCG CGTTGCGTTA ATTACACTCA ATGGAGTGAG TAATCCGTGG GGTCCGAAAT GTGAAATACG
```

FIG.32Z-4

```
        mspI                                  aciI
        hpaII                              bsrBI                                    aluI       nlaIII
5061 TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA ATTTCACACA GGAAACAGCT ATGACCATGA
     AAGGCCGAGC ATACAACACA CCTTAACACT CGCCTATTGT TAAAGTGTGT CCTTTGTCGA TACTGGTACT

          tru9I
          mseI
         aseI/asnI/vspI
       xmnI
       asp700
5131 TTACGAATTA A
     AATGCTTAAT T

>length: 5141
```

FIG.32Z-5

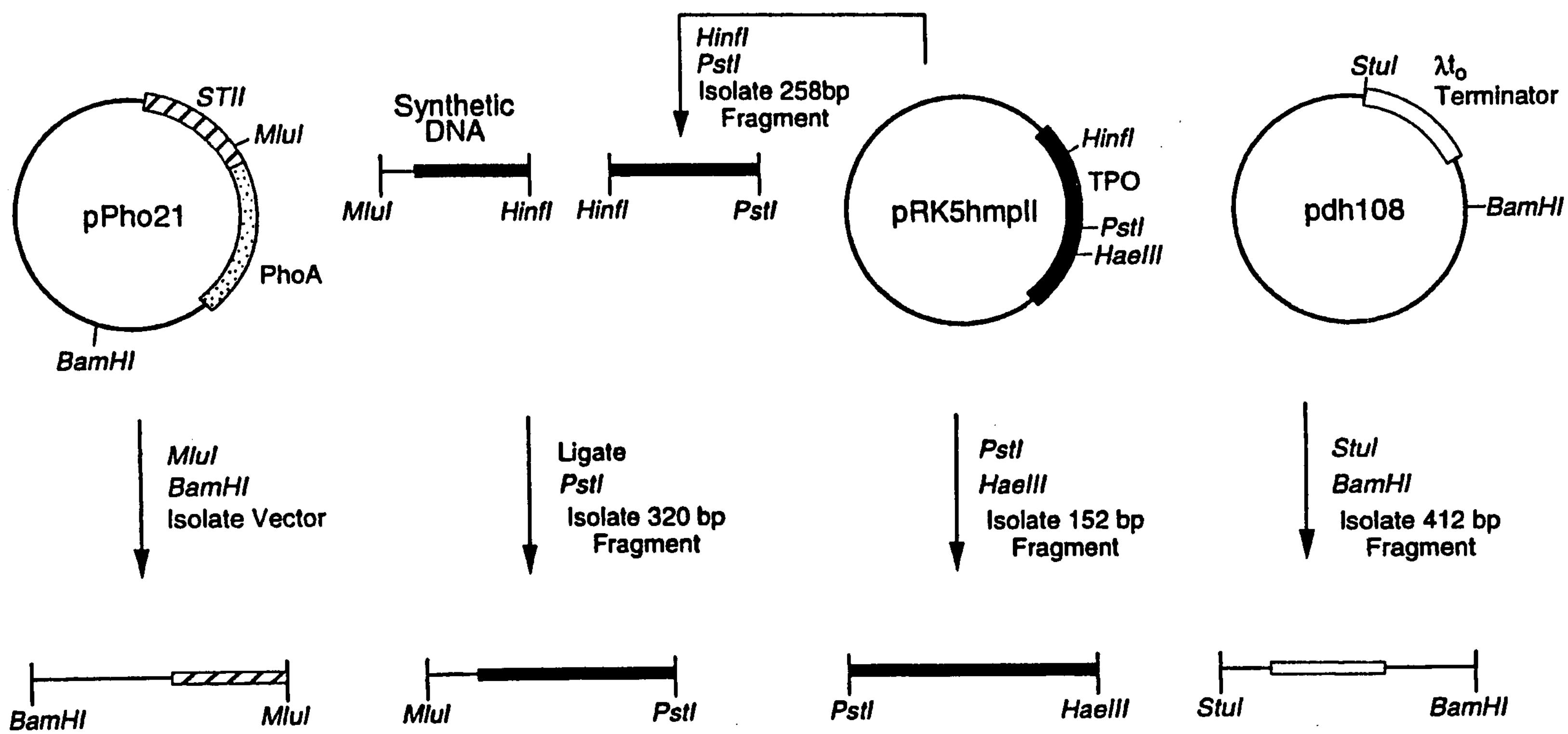

pPho21
STII
MluI
PhoA
BamHI
MluI
BamHI
Isolate Vector
BamHI
MluI
Synthetic DNA
MluI
HinfI
HinfI
PstI
Isolate 258bp Fragment
HinfI
PstI
Ligate
PstI
Isolate 320 bp Fragment
MluI
PstI
pRK5hmplI
HinfI
TPO
PstI
HaeIII
PstI
HaeIII
Isolate 152 bp Fragment
PstI
HaeIII
pdh108
StuI
λt_o Terminator
BamHI
StuI
BamHI
Isolate 412 bp Fragment
StuI
BamHI

FIG.33A

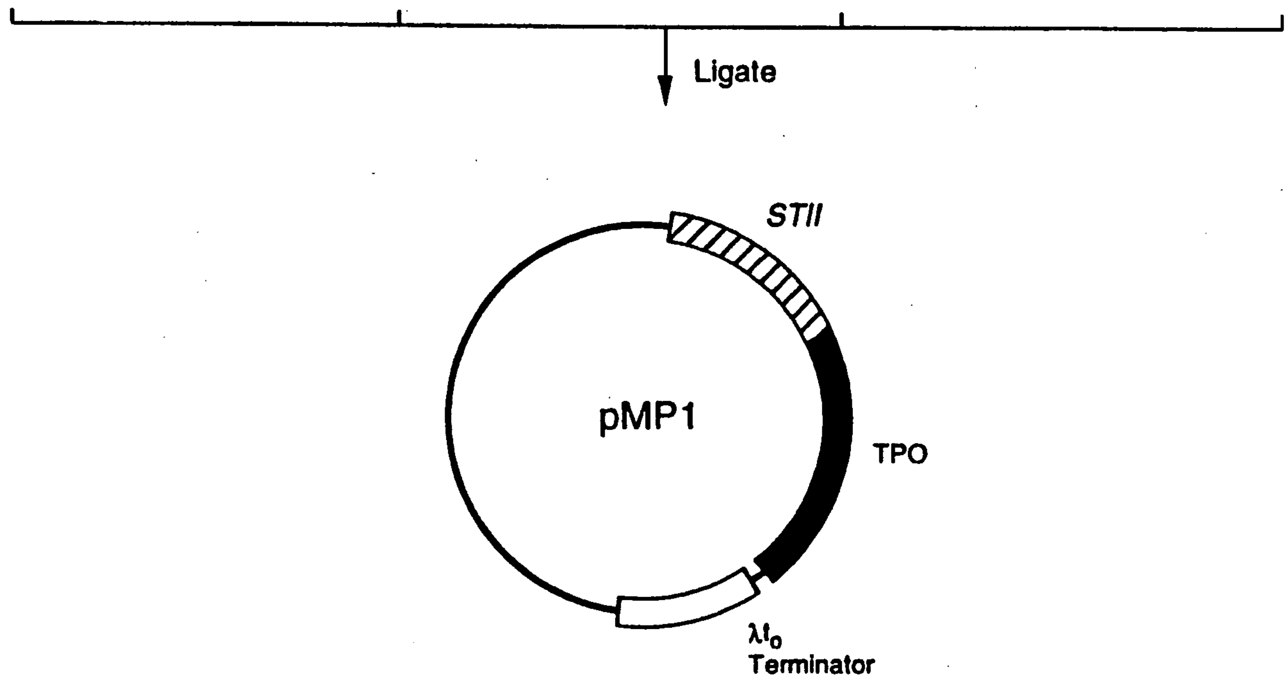
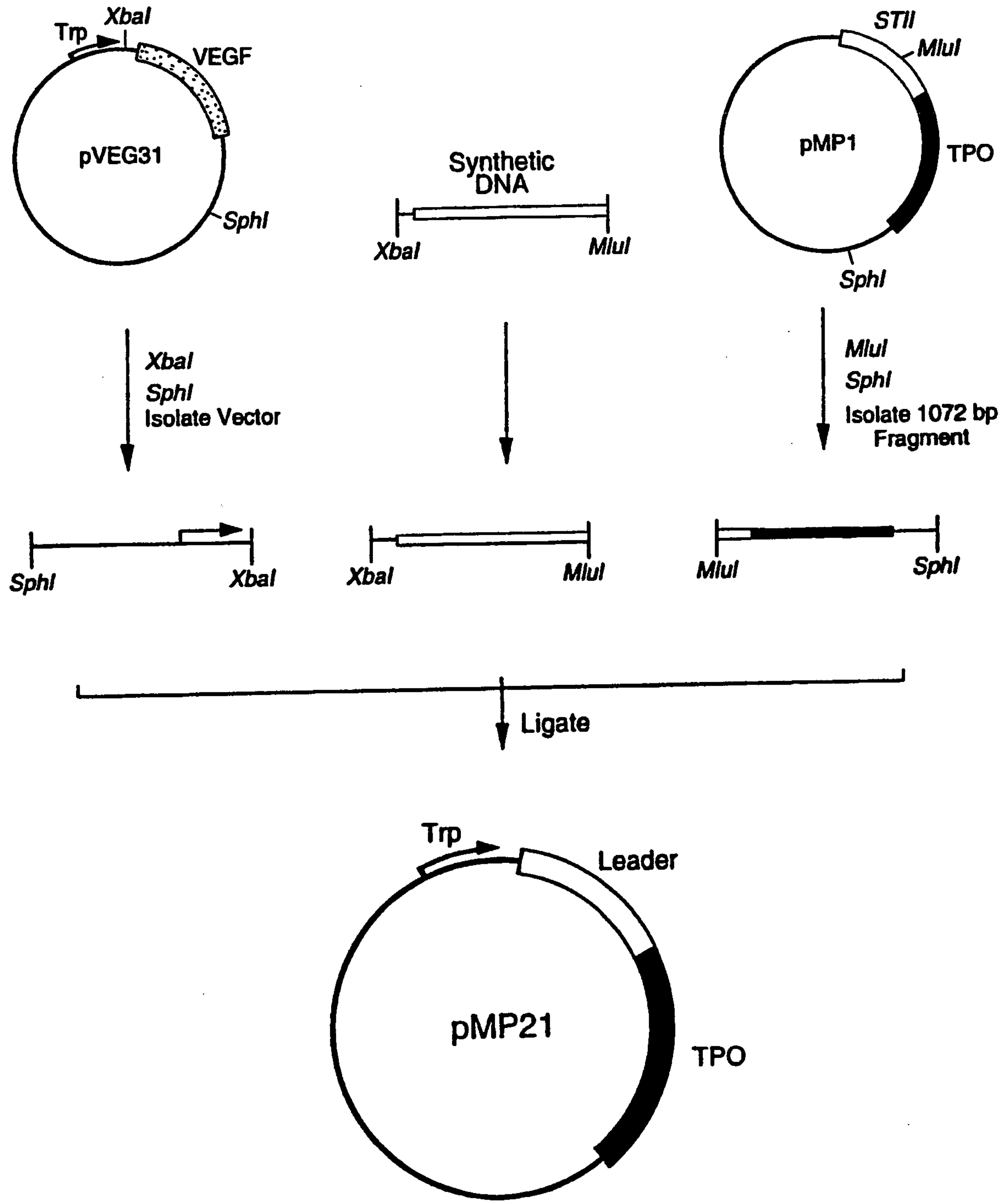


FIG.33B



Trp
XbaI
VEGF
pVEG31
SphI
Synthetic DNA
XbaI
MluI
STII
MluI
pMP1
TPO
SphI
XbaI
SphI
Isolate Vector
MluI
SphI
Isolate 1072 bp Fragment
SphI
XbaI
XbaI
MluI
MluI
SphI
Ligate
Trp
Leader
pMP21
TPO

FIG.34

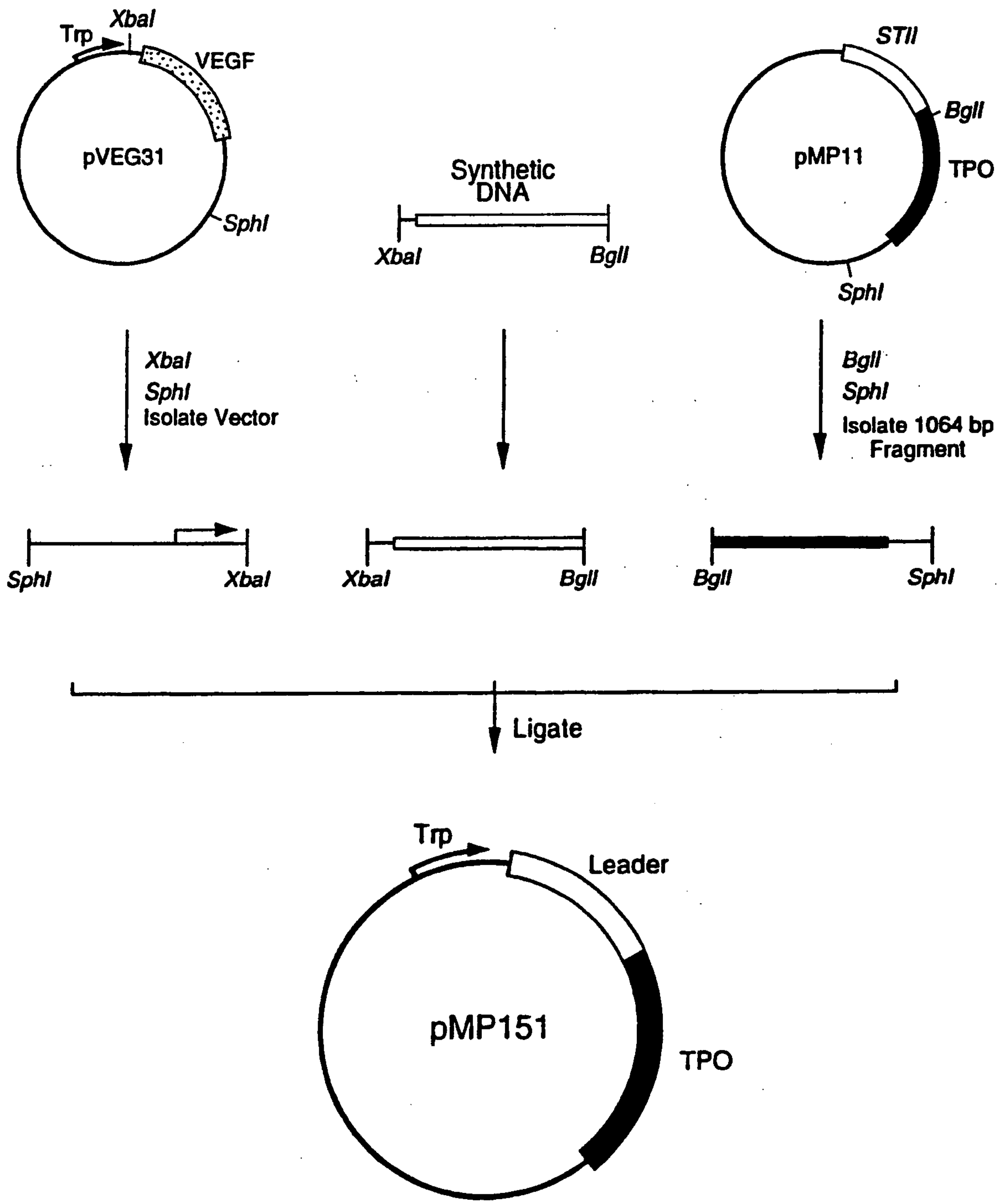
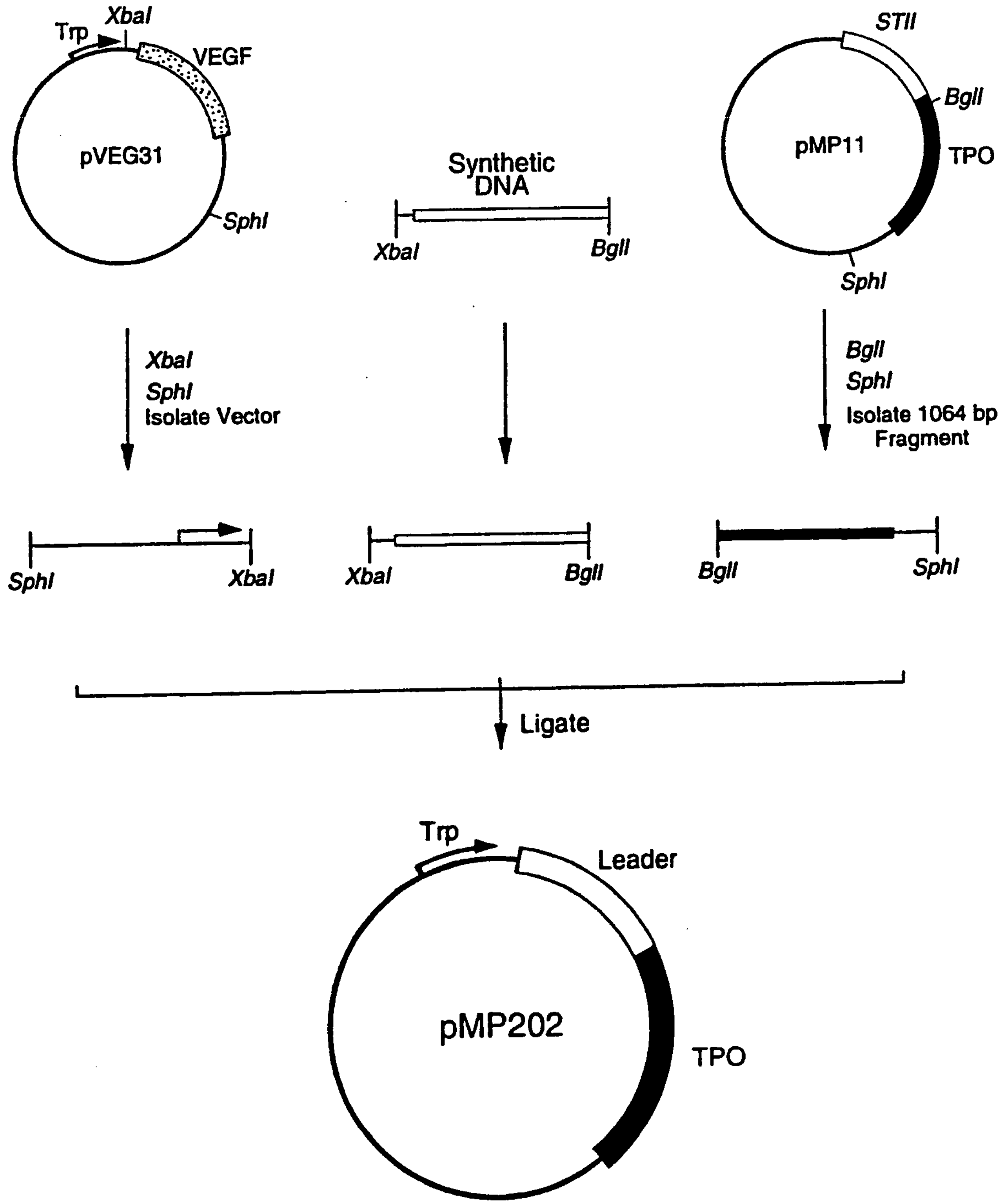

Trp
XbaI
VEGF
pVEG31
SphI
Synthetic DNA
XbaI
BglI
STII
BglI
pMP11
TPO
SphI
XbaI
SphI
Isolate Vector
BglI
SphI
Isolate 1064 bp Fragment
SphI
XbaI
XbaI
BglI
BglI
SphI
Ligate
Trp
Leader
pMP151
TPO

FIG.35



Trp
XbaI
VEGF
pVEG31
SphI
Synthetic DNA
XbaI
BglI
STII
BglI
pMP11
TPO
SphI
XbaI
SphI
Isolate Vector
BglI
SphI
Isolate 1064 bp Fragment
SphI
XbaI
XbaI
BglI
BglI
SphI
Ligate
Trp
Leader
pMP202
TPO

FIG.36

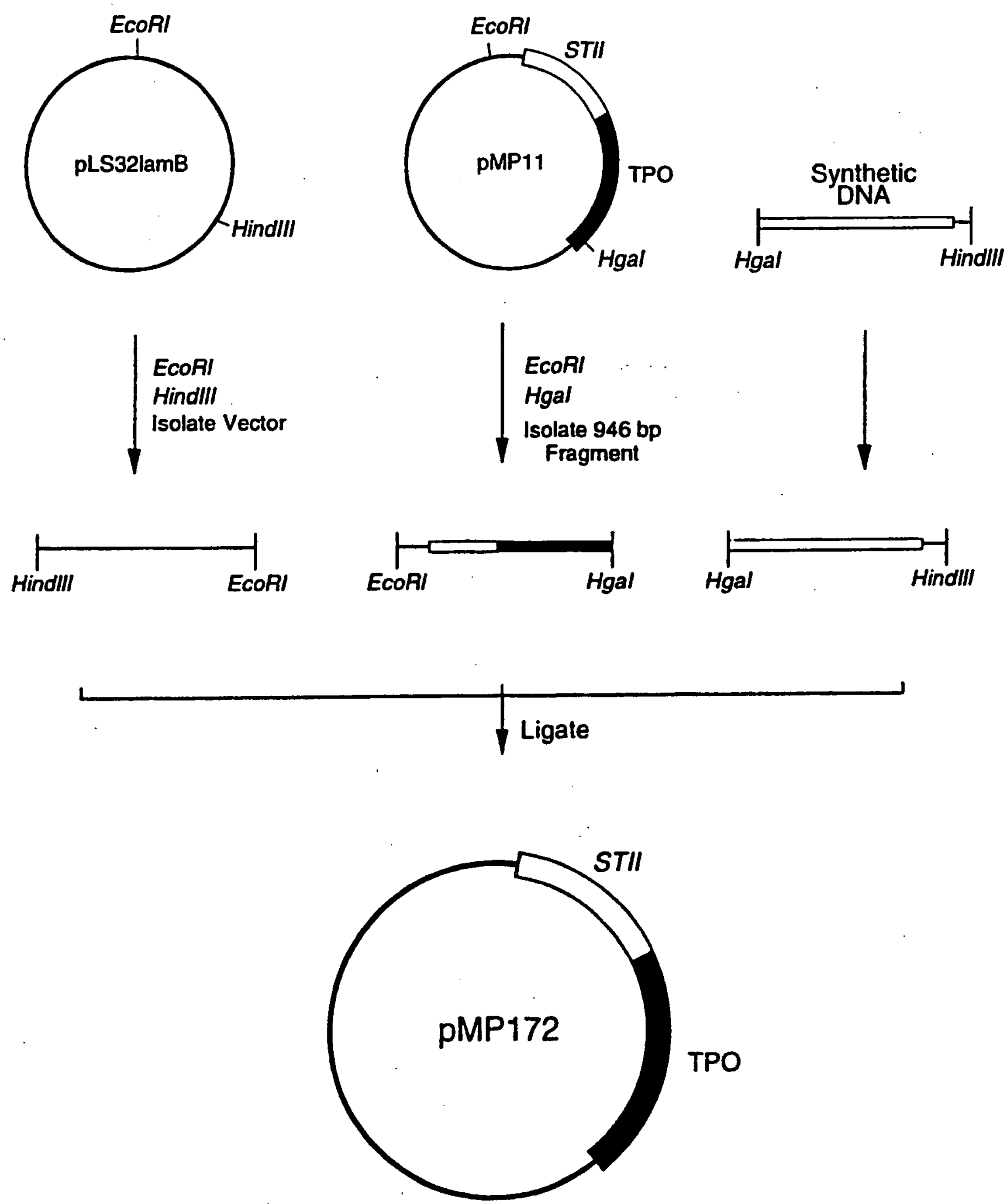
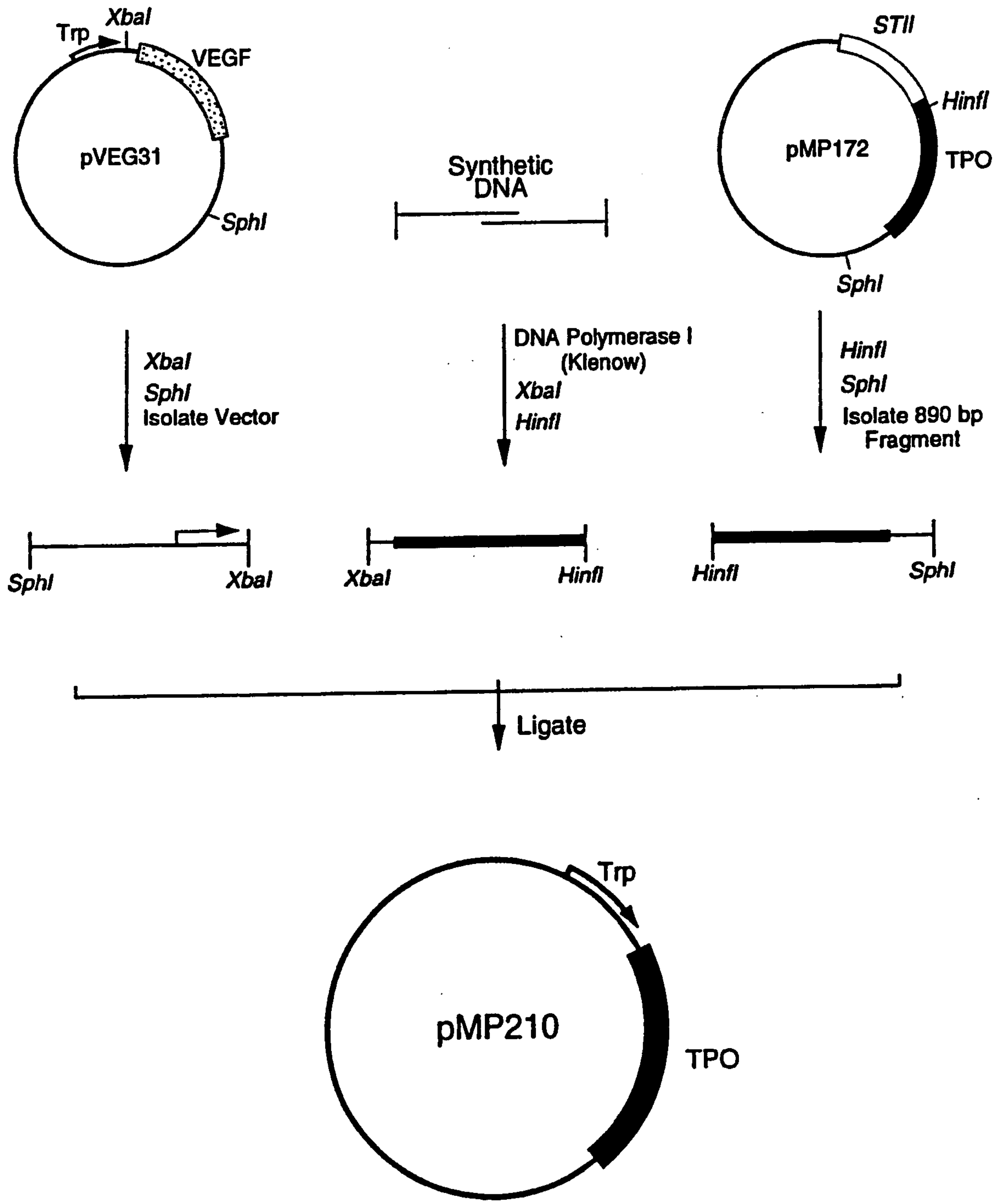

EcoRI
pLS32lamB
HindIII
EcoRI
STII
pMP11
TPO
HgaI
Synthetic DNA
HgaI
HindIII
EcoRI
HindIII
Isolate Vector
EcoRI
HgaI
Isolate 946 bp Fragment
HindIII
EcoRI
EcoRI
HgaI
HgaI
HindIII
Ligate
STII
pMP172
TPO

FIG.37



Trp
XbaI
VEGF
pVEG31
SphI
Synthetic DNA
STII
HinfI
pMP172
TPO
SphI
XbaI
SphI
Isolate Vector
DNA Polymerase I (Klenow)
XbaI
HinfI
HinfI
SphI
Isolate 890 bp Fragment
SphI
XbaI
XbaI
HinfI
HinfI
SphI
Ligate
Trp
pMP210
TPO

FIG.38

	Met	Ser	Pro	Ala	Pro	Pro	Ala
MP210 Bank	ATG	TCN	CCN	GCN	CCN	CCN	GCN
MP210-1	ATG	TCT	CCA	GCG	CCG	CCA	GCG
MP210-T8	ATG	TCG	CCT	GCT	CCA	CCT	GCT
MP210-21	ATG	TCG	CCA	GCG	CCA	CCA	GCC
MP210-24	ATG	TCC	CCA	GCC	CCA	CCC	GCA
MP210-25	ATG	TCG	CCA	GCG	CCG	CCA	GCG

FIG.39

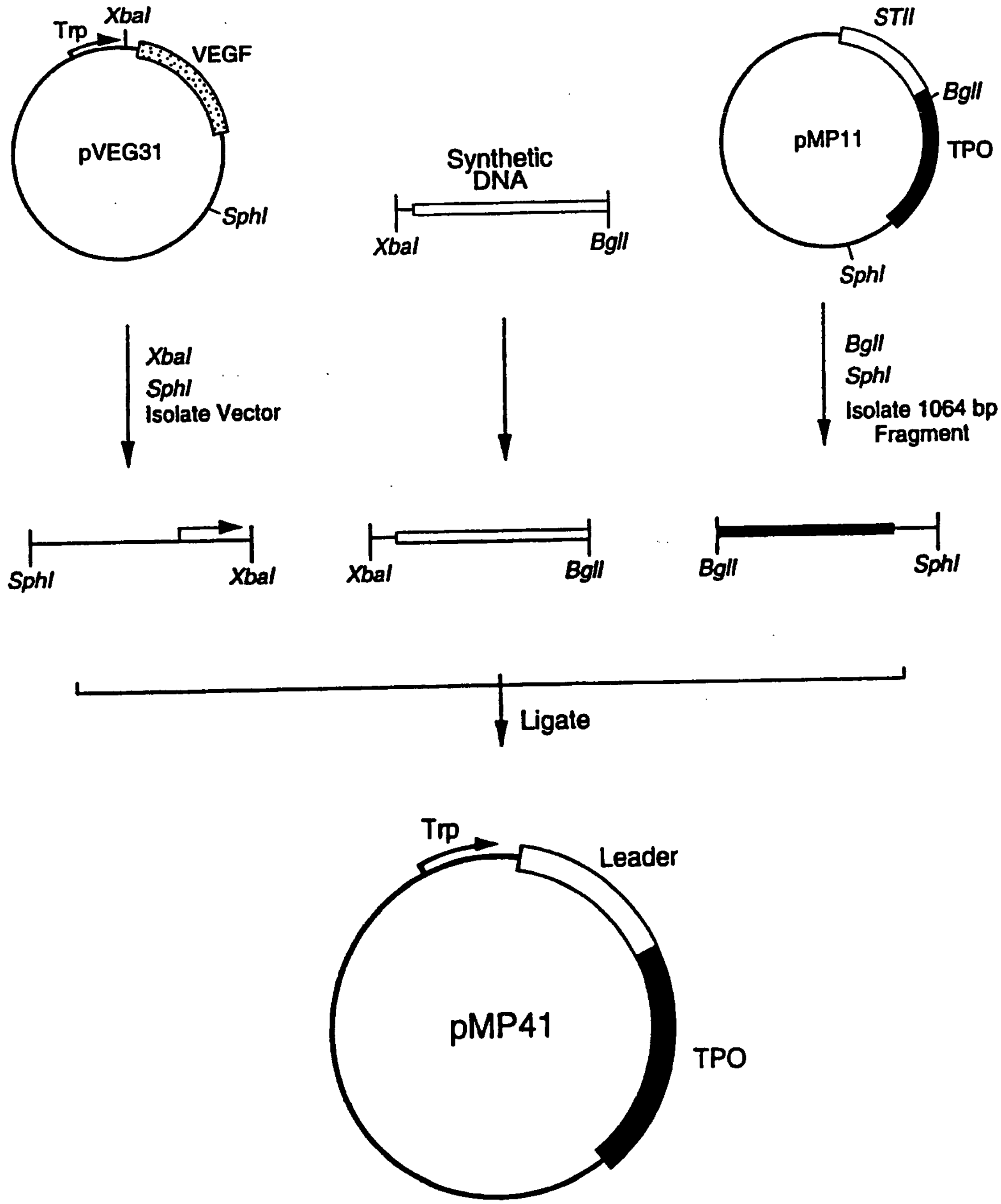

Trp
XbaI
VEGF
pVEG31
SphI
Synthetic
DNA
XbaI
BglI
STII
BglI
pMP11
TPO
SphI
XbaI
SphI
Isolate Vector
BglI
SphI
Isolate 1064 bp
Fragment
SphI
XbaI
XbaI
BglI
BglI
SphI
Ligate
Trp
Leader
pMP41
TPO

FIG.40

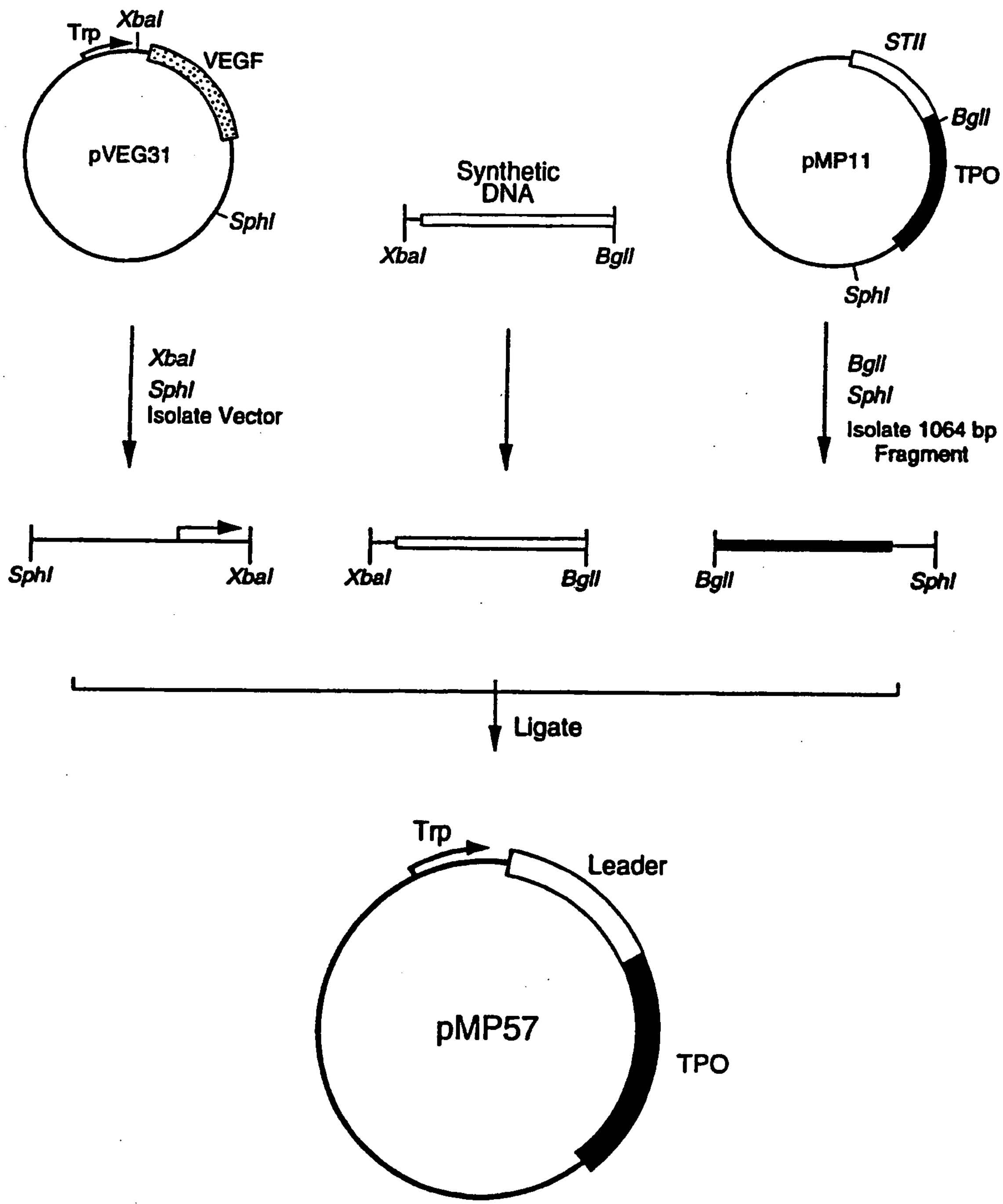
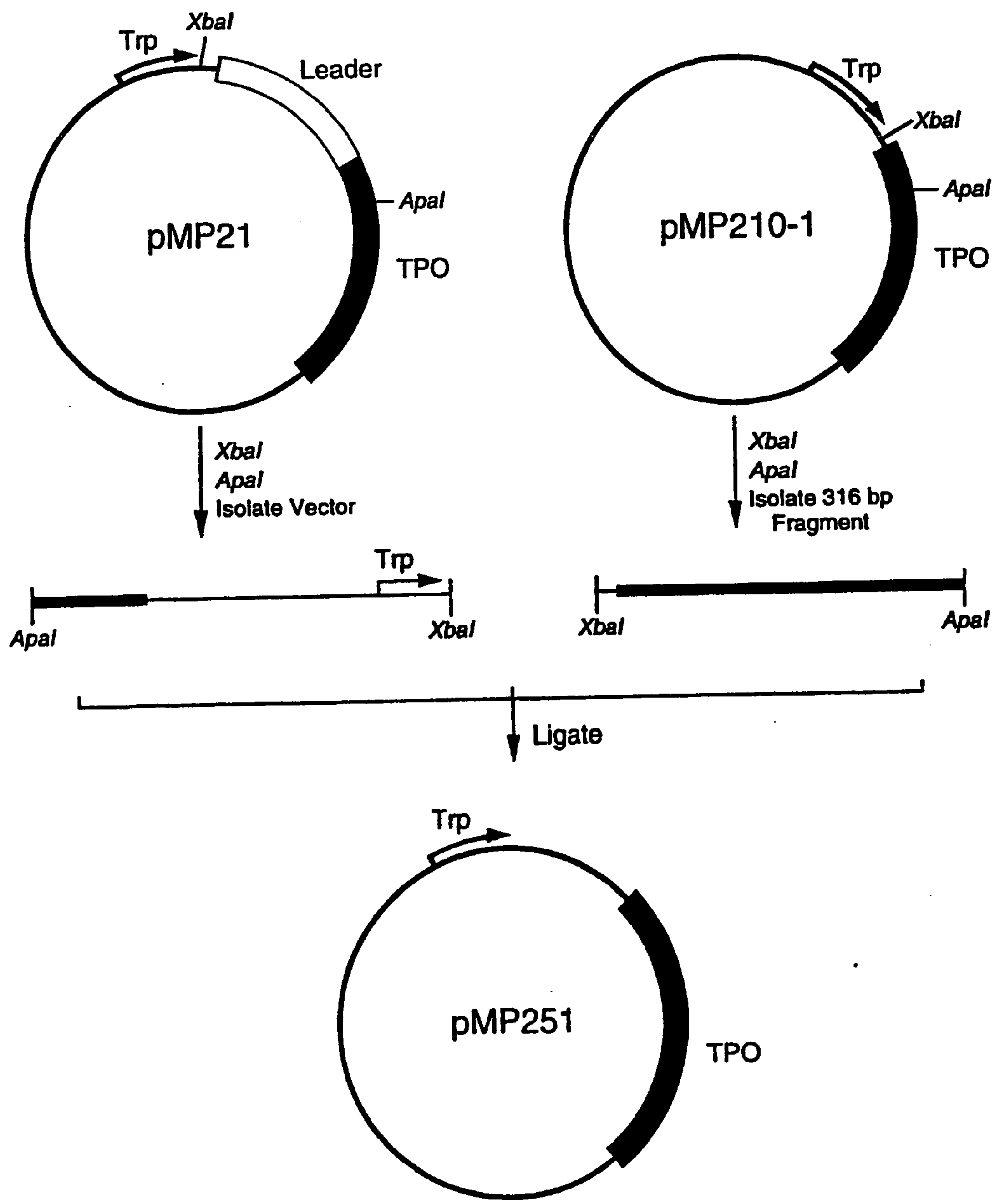

Trp
XbaI
VEGF
pVEG31
SphI
Synthetic DNA
XbaI
BglI
STII
BglI
pMP11
TPO
SphI
XbaI
SphI
Isolate Vector
BglI
SphI
Isolate 1064 bp Fragment
SphI
XbaI
XbaI
BglI
BglI
SphI
Ligate
Trp
Leader
pMP57
TPO

FIG.41



Trp
XbaI
Leader
ApaI
pMP21
TPO
Trp
XbaI
ApaI
pMP210-1
TPO
XbaI
ApaI
Isolate Vector
XbaI
ApaI
Isolate 316 bp
Fragment
Trp
ApaI
XbaI
XbaI
ApaI
Ligate
Trp
pMP251
TPO

FIG.42

THROMBOPOIETIN

FIELD OF THE INVENTION

This invention relates to the isolation, purification and recombinant or chemical synthesis of proteins that influence survival, proliferation, differentiation or maturation of hematopoietic cells, especially platelet progenitor cells. This invention specifically relates to the cloning and expression of nucleic acids encoding a protein ligand capable of binding to and activating *mpl*, a member of the cytokine receptor superfamily. This invention further relates to the use of these proteins alone or in combination with other cytokines to treat immune or hematopoietic disorders including thrombocytopenia.

BACKGROUND OF THE INVENTION

I. The Hematopoietic System

The hematopoietic system produces the mature highly specialized blood cells known to be necessary for survival of all mammals. These mature cells include: erythrocytes, specialized to transport oxygen and carbon dioxide, T- and B-lymphocytes, responsible for cell- and antibody-mediated immune responses, platelets or thrombocytes, specialized to form blood clots, and granulocytes and macrophages, specialized as scavengers and as accessory cells to combat infection. Granulocytes are further subdivided into: neutrophils, eosinophils, basophils and mast cells, specialized cell types having discrete functions Remarkably, all of these specialized mature blood cells are derived from a single common primitive cell type, referred to as the pluripotent (or totipotent) stem cell, found primarily in bone marrow (Dexter *et al.*, *Ann. Rev. Cell Biol.*, 3:423-441 [1987]).

The mature highly specialized blood cells must be produced in large numbers continuously throughout the life of a mammal. The vast majority of these specialized blood cells are destined to remain functionally active for only a few hours to weeks (Cronkite *et al.*, *Blood Cells*, 2:263-284 [1976]). Thus, continuous renewal of the mature blood cells, the primitive stem cells themselves, as well as any intermediate or lineage-committed progenitor cell lines lying between the primitive and mature cells, is necessary in order to maintain the normal steady state blood cell needs of the mammal.

At the heart of the hematopoietic system lies the pluripotent stem cell(s). These cells are relatively few in number and undergo self-renewal by proliferation to produce daughter stem cells or are transformed, in a series of differentiation steps,

into increasingly mature lineage-restricted progenitor cells, ultimately forming the highly specialized mature blood cell(s).

For example, certain multipotent progenitor cells, referred to as CFC-Mix, derived from stem cells undergo proliferation (self-renewal) and development to produce colonies containing all the different myeloid cells; erythrocytes, neutrophils, megakaryocytes (predecessors of platelets), macrophages, basophils, eosinophils, and mast cells. Other progenitor cells of the lymphoid lineage undergo proliferation and development into T-cells and B-cells.

Additionally, between the CFC-Mix progenitor cells and myeloid cells lie another rank of progenitor cells of intermediate commitment to their progeny. These lineage-restricted progenitor cells are classified on the basis of the progeny they produce. Thus, the known immediate predecessors of the myeloid cells are: erythroid colony-forming units (CFU-E) for erythrocytes, granulocyte/macrophage colony-forming cells (GM-CFC) for neutrophils and macrophages, megakaryocyte colony-forming cells (Meg-CFC) for megakaryocytes, eosinophil colony-forming cells (Eos-CFC) for eosinophils, and basophil colony-forming cells (Bas-CFC) for mast cells. Other intermediate predecessor cells between the pluripotent stem cells and mature blood cells are known (see below) or will likely be discovered having varying degrees of lineage-restriction and self-renewal capacity.

The underlying principal of the normal hematopoietic cell system appears to be decreased capacity for self-renewal as multipotency is lost and lineage-restriction and maturity is acquired. Thus, at one end of the hematopoietic cell spectrum lies the pluripotent stem cell possessing the capacity for self-renewal and differentiation into all the various lineage-specific committed progenitor cells. This capacity is the basis of bone marrow transplant therapy where primitive stem cells repopulate the entire hematopoietic cell system. At the other end of the spectrum lie the highly lineage-restricted progenitors and their progeny which have lost the ability of self-renewal but have acquired mature functional activity.

The proliferation and development of stem cells and lineage-restricted progenitor cells is carefully controlled by a variety of hematopoietic growth factors or cytokines. The role of these growth factors *in vivo* is complex and incompletely understood. Some growth factors, such as interleukin-3 (IL-3), are capable of stimulating both multipotent stem cells as well as committed progenitor cells of several lineages, including for example, megakaryocytes. Other factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) was initially thought to be restricted in its action to GM-CFC's. Later, however, it was discovered GM-CSF also influenced the proliferation and development of *interalia* megakaryocytes. Thus, IL-3 and GM-CSF were found to have overlapping biological activities, although with

differing potency. More recently, both interleukin-6 (IL-6) and interleukin-11 (IL-11), while having no apparent influence on meg-colony formation alone, act synergistically with IL-3 to stimulate maturation of megakaryocytes (Yonemura *et al.*, *Exp. Hematol.*, **20**:1011-1016 [1992]).

Thus, hematopoietic growth factors may influence growth and differentiation of one or more lineages, may overlap with other growth factors in affecting a single progenitor cell line, or may act synergistically with other factors.

It also appears that hematopoietic growth factors can exhibit their effect at different stages of cell development from the totipotent stem cell through various committed lineage-restricted progenitors to the mature blood cell. For example, erythropoietin (epo) appears to promote proliferation only of mature erythroid progenitor cells. IL-3 appears to exert its effect earlier influencing primitive stem cells and intermediate lineage-restricted progenitor cells. Other growth factors such as stem cell factor (SCF) may influence even more primitive cell development.

It will be appreciated from the foregoing that novel hematopoietic growth factors that affect survival, proliferation, differentiation or maturation of any of the blood cells or predecessors thereof would be useful, especially to assist in the re-establishment of a diminished hematopoietic system caused by disease or after radiation- or chemo-therapy.

II. Megakaryocytopoiesis - Platelet Production

Regulation of megakaryocytopoiesis and platelet production has been reviewed by: Mazur, *Exp. Hematol.*, 15:248 [1987] and Hoffman, *Blood*, 74:1196-1212 [1989]. Briefly, bone marrow pluripotent stem cells differentiate into megakaryocytic, erythrocytic, and myelocytic cell lines. It is believed there is a hierarchy of committed megakaryocytic progenitor cells between stem cells and megakaryocytes. At least three classes of megakaryocytic progenitor cells have been identified, namely; burst forming unit megakaryocytes (BFU-MK), colony-forming unit megakaryocytes (CFU-MK), and light density megakaryocyte progenitor cells (LD-CFU-MK). Megakaryocytic maturation itself is a continuum of development that has been separated into stages based on standard morphologic criteria. The earliest recognizable member of the megakaryocyte (MK or meg) family are the megakaryoblasts. These cells are initially 20 to 30 μm in diameter having basophilic cytoplasm and a slightly irregular nucleus with loose, somewhat reticular chromatin and several nucleoli. Later, megakaryoblasts may contain up to 32 nuclei (ployploid), but the cytoplasm remains sparse and immature. As maturation proceeds, the nucleus becomes more lobulate and pyknotic, the cytoplasm increases in quantity and becomes more acidophilic and granular. The most mature cells of this family may give the

appearance of releasing platelets at their periphery. Normally, less than 10% of megakaryocytes are in the blast stage and more than 50% are mature. Arbitrary morphologic classifications commonly applied to the megakaryocyte series are megakaryoblast for the earliest form; promegakaryocyte or basophilic megakaryocyte for the intermediate form; and mature (acidophilic, granular, or platelet-producing) megakaryocyte for the late forms. The mature megakaryocyte extends filaments of cytoplasm into sinusoidal spaces where they detach and fragment into individual platelets (Williams *et al.*, *Hematology*, 1972).

Megakaryocytopoiesis is believed to involve several regulatory factors (Williams *et al.*, *Br. J. Haematol.*, 52:173 [1982] and Williams *et al.*, *J. Cell Physiol.*, 110:101 [1982]). The early level of megakaryocytopoiesis is postulated as being mitotic, concerned with cell proliferation and colony initiation from CFU-MK but is not affected by platelet count (Burstein *et al.*, *J. Cell Physiol.*, 109:333 [1981] and Kimura *et al.*, *Exp. Hematol.*, 13:1048 [1985]). The later stage of maturation is non-mitotic, involved with nuclear polyploidization and cytoplasmic maturation and is probably regulated in a feedback mechanism by peripheral platelet number (Odell *et al.*, *Blood*, 48:765 [1976] and Ebbe *et al.*, *Blood*, 32:787 [1968]).

The existence of a distinct and specific megakaryocyte colony-stimulating factor (MK-CSF) has been disputed (Mazur, *Exp. Hematol.*, 15:340-350 [1987]) However most authors believe that a process so vital to survival as platelet production would be regulated by cytokine(s) exclusively responsible for this process. The hypothesis that megakaryocyte/platelet specific cytokine(s) exist has provided the basis for more than 30 years of search - but to date no such cytokine has been purified, sequenced and established by assay as a unique MK-CSF (TPO).

Although it has been reported that MK-CSF's have been partly purified from experimentally produced thrombocytopenia (Hill *et al.*, *Exp. Hematol.*, 14:752 [1986]) and human embryonic kidney conditioned medium [CM] (McDonald *et al.*, *J. Lab. Clin. Med.*, 85:59 [1975]) and in man from a plastic anemia and idiopathic thrombocytopenic purpura urinary extracts (Kawakita *et al.*, *Blood*, 6:556 [1983]) and plasma (Hoffman *et al.*, *J. Clin. Invest.*, 75:1174 [1985]), their physiological function is as yet unknown in most cases.

The conditioned medium of pokeweed mitogen-activated spleen cells (PWM-SpCM) and the murine myelomonocyte cell line WEHI-3 (WEHI-3CM) have been used as megakaryocyte potentiators. PWM-SpCM contains factors enhancing CFU-MK growth (Metcalf *et al.*, *Pro. Natl. Acad. Sci., USA*, 72:1744-1748 [1975]; Quesenberry *et al.*, *Blood*, 65:214 [1985]; and Iscove, N.N., in *Hematopoietic Cell Differentiation, ICN-UCLA Symposia on Molecular and Cellular Biology*, Vol. 10, Golde

et al., eds. [New York, Academy Press] pp 37-52 [1978]), one of which is interleukin-3 (IL-3), a multilineage colony stimulating factor (multi-CSF [Burstein, *Blood Cells*, **11**:469 [1986]). The other factors in this medium have not yet been identified and isolated. WEHI-3 is a murine myelomonocytic cell line secreting relatively large amounts of IL-3 and smaller amounts of GM-CSF. IL-3 has been found to potentiate the growth of a wide range of hematopoietic cells (Ihle *et al.*, *J. Immunol.*, **13**:282 [1983]). IL-3 has also been found to synergize with many of the known hematopoietic hormones or growth factors (Bartelmez *et al.*, *J. Cell Physiol.*, **122**:362-369 [1985] and Warren *et al.*, *Cell*, **46**:667-674 [1988]), including both erythropoietin (EPO) and interleukin-1 (IL-1), in the induction of very early multipotential precursors and the formation of very large mixed hematopoietic colonies.

Other sources of megakaryocyte potentiators have been found in the conditioned media of murine lung, bone, macrophage cell lines, peritoneal exudate cells and human embryonic kidney cells. Despite certain conflicting data (Mazur, *Exp. Hematol.*, **15**:340- 350 [1987]), there is some evidence (Geissler *et al.*, *Br. J. Haematol.*, **60**:233-238 [1985]) that activated T lymphocytes rather than monocytes play an enhancing role in megakaryocytopoiesis. These findings suggest that activated T-lymphocyte secretions such as interleukins may be regulatory factors in MK development (Geissler *et al.*, *Exp Hematol.*, **15**:845-853 [1987]). A number of studies on megakaryocytopoiesis with purified erythropoietin EPO (Vainchenker *et al.*, *Blood*, **54**:940 [1979]; McLeod *et al.*, *Nature*, **261**:492-4 [1976]; and Williams *et al.*, *Exp. Hematol.*, **12**:734 [1984]) indicate that this hormone has an enhancing effect on MK colony formation. This has also been demonstrated in both serum-free and serum-containing cultures and in the absence of accessory cells (Williams *et al.*, *Exp. Hematol.*, **12**:734 [1984]). EPO was postulated to be involved more in the single and two-cell stage aspects of megakaryocytopoiesis as opposed to the effect of PWM-SpCM which was involved in the four-cell stage of megakaryocyte development. The interaction of all these factors on both early and late phases of megakaryocyte development remains to be elucidated.

Data produced from several laboratories suggests that the only multi-lineage factors that individually have MK-colony stimulating activity are GM-CSF and IL-3 and, to a lesser extent, the B-cell stimulating factor IL-6 (Ikebuchi *et al.*, *Proc. Natl. Acad. Sci. USA*, **84**:9035 [1987]). More recently, several authors have reported that IL-11 and leukemia inhibitory factor (LIF) act synergistically with IL-3 to increase megakaryocyte size and ploidy (Yonemura *et al.*, *British Journal of Hematology*, **84**:16-23 [1993]; Burstein *et al.*, *J. Cell. Physiol.*, **153**:305-312 [1992]; Metcalf *et al.*, *Blood*, **76**:50-56 [1990]; Metcalf *et al.*, *Blood*, **77**:2150-2153 [1991];

Bruno *et al.*, *Exp. Hematol.*, **19**:378-381 [1991]; and Yonemura *et al.*, *Exp. Hematol.*, **20**:1011-1016 [1992]).

Other documents of interest include: Eppstein *et al.*, U.S. Patent No. 4,962,091; Chong, U.S. Patent No. 4,879,111; Fernandes *et al.*, U.S. Patent No. 4,604,377; Wissler *et al.*, U.S. Patent No. 4,512,971; Gottlieb, U.S. Patent No. 4,468,379; Bennett *et al.*, U.S. Patent No 5,215,895; Kogan *et al.*, U.S. Patent No. 5,250,732; Kimura *et al.*, *Eur. J. Immunol.*, **20(9)**:1927-1931 [1990]; Secor *et al.*, *J. of Immunol.*, **144(4)**:1484-1489 [1990]; Warren *et al.*, *J. of Immunol.*, **140(1)**:94-99 [1988]; Warren *et al.*, *Exp. Hematol.*, **17(11)**:1095-1099 [1989]; Bruno *et al.*, *Exp. Hematol.*, **17(10)**:1038-1043 [1989]; Tanikawa *et al.*, *Exp. Hematol.*, **17(8)**:883-888 [1989], Koike *et al.*, *Blood*, **75(12)**:2286-2291 [1990]; Lotem, *Blood*, **75(5)**:1545-1551 [1989]; Rennick *et al.*, *Blood*, **73(7)**:1828-1835 [1989]; and Clutterbuck *et al.*, *Blood*, **73(6)**:1504-1512 [1989].

III. Thrombocytopenia

Platelets are critical elements of the blood clotting mechanism. Depletion of the circulating level of platelets, called thrombocytopenia, occurs in various clinical conditions and disorders. Thrombocytopenia is commonly defined as a platelet count below 150 X 10^9 per liter. The major causes of thrombocytopenia can be broadly divided into three categories on the basis of platelet life span, namely: (1) impaired production of platelets by the bone marrow, (2) platelet sequestration in the spleen (splenomegaly), or (3) increased destruction of platelets in the peripheral circulation (*e.g.*, autoimmune thrombocytopenia or chemo- and radiation-therapy). Additionally, in patients receiving large volumes of rapidly administered platelet-poor blood products, thrombocytopenia may develop due to dilution.

The clinical bleeding manifestations of thrombocytopenia depend on the severity of thrombocytopenia, its cause, and possible associated coagulation defects. In general, patients with platelet counts between 20 and 100 X 10^9 per liter are at risk of excessive post traumatic bleeding, while those with platelet counts below 20 X 10^9 per liter may bleed spontaneously. These latter patients are candidates for platelet transfusion with attendant immune and viral risk. For any given degree of thrombocytopenia, bleeding tends to be more severe when the cause is decreased production rather than increased destruction of platelets. In the latter situation, accelerated platelet turnover results in the circulation of younger, larger and hemostatically more effective platelets. Thrombocytopenia may result from a variety of disorders briefly described below. A more detailed description may be found in Schafner, A. I., "Thrombocytopenia and Disorders of Platelet Function," *Internal*

Medicine, 3rd Ed., John J. Hutton *et al.*, Eds., Little Brown and Co., Boston/Toronto/ London [1990].

(a) Thrombocytopenia due to impaired platelet production

Causes of congenital thrombocytopenia include constitutional aplastic anemia (Fanconi syndrome) and congenital amegakaryocytic thrombocytopenia, which may be associated with skeletal malformations. Acquired disorders of platelet production are caused by either hypoplasia of megakaryocytes or ineffective thrombopoiesis. Megakaryocytic hypoplasia can result from a variety of conditions, including marrow aplasia (including idiopathic forms or myelosuppression by chemotherapeutic agents or radiation therapy), myelfibrosis, leukemia, and invasion of the bone marrow by metastatic tumor or granulomas. In some situations, toxins, infectious agents, or drugs may interfere with thrombopoiesis relatively selectively; examples include transient thrombocytopenias caused by alcohol and certain viral infections and mild thrombocytopenia associated with the administration of thiazide diuretics. Finally, ineffective thrombopoiesis secondary to megaloblastic processes (folate or B_{12} deficiency) can also cause thrombocytopenia, usually with coexisting anemia and leukopenia

Current treatment of thrombocytopenias due to decreased platelet production depends on identification and reversal of the underlying cause of the bone marrow failure. Platelet transfusions are usually reserved for patients with serious bleeding complications, or for coverage during surgical procedures, since isoimmunization may lead to refractoriness to further platelet transfusions. Mucosal bleeding resulting from severe thrombocytopenia may be ameliorated by the oral or intravenous administration of the antifibrinolytic agents. Thrombotic complications may develop, however, if antifibrinolytic agents are used in patients with disseminated intravascular coagulation (DIC).

(b) Thrombocytopenia due to splenic sequestration

Splenomegaly due to any cause may be associated with mild to moderate thrombocytopenia. This is a largely passive process (hypersplenism) of splenic platelet sequestration, in contrast to the active destruction of platelets by the spleen in cases of immunomediated thrombocytopenia discussed below. Although the most common cause of hypersplenism is congestive splenomegaly from portal hypertension due to alcoholic cirrhosis, other forms of congestive, infiltrative, or lymphoproliferative splenomegaly are also associated with thrombocytopenia. Platelet counts generally do not fall below 50 X 10^9 per liter as a result of hypersplenism alone.

(c) Thrombocytopenia due to nonimmune-mediated platelet destruction

Thrombocytopenia can result from the accelerated destruction of platelets by various nonimmunologic processes. Disorders of this type include disseminated intravascular coagulation, prosthetic intravascular devices, extra corporeal circulation of the blood, and thrombotic microangiopathies such as thrombotic thrombocytic purpura. In all of these situations, circulating platelets that are exposed to either artificial surfaces or abnormal vascular intima either are consumed at these sites or are damaged and then prematurely cleared by the reticuloendothelial system. Disease states or disorders in which disseminated intravascular coagulation (DIC) may arise are set forth in greater detail in Braunwald *et al.* (eds), *Harrison's Principles of Internal Medicine*, 11th Ed., p.1478, McGraw Hill [1987]. Intravascular prosthetic devices, including cardiac valves and intra-aortic balloons can cause a mild to moderate destructive thrombocytopenia and transient thrombocytopenia in patients undergoing cardiopulmonary bypass or hemodialysis may result from consumption or damage of platelets in the extra corporeal circuit.

(d) Drug-induced immune thrombocytopenia

More than 100 drugs have been implicated in immunologically mediated thrombocytopenia. However, only quinidine quinine, gold, sulfonamides, cephalothin, and heparin have been well characterized. Drug-induced thrombocytopenia is frequently very severe and typically occurs precipitously within days while patients are taking the sensitizing medication.

(e) Immune (autoimmune) thrombocytopenic purpura (ITP)

ITP in adults is a chronic disease characterized by autoimmune platelet destruction. The autoantibody is usually IgG although other immunoglobulins have also been reported. Although the autoantibody of ITP has been found to be associated with platelet membrane $GPII_bIII_a$, the platelet antigen specificity has not been identified in most cases. Extravascular destruction of sensitized platelets occurs in the reticuloendothelial system of the spleen and liver. Although over one-half of all cases of ITP are idiopathic, many patients have underlying rheumatic or autoimmune diseases (*e.g.*, systemic lupus erythematosus) or lymphoproliferative disorders (*e.g.*, chronic lymphocytic leukemia).

(f) HIV-Induced ITP

ITP is an increasingly common complication of HIV infection (Morris *et al.*, *Ann. Intern. Med.*, 96:714-717 [1982]), and can occur at any stage of the disease progression, both in patients diagnosed with the Acquired Immune Deficiency Syndrome (AIDS), those with AIDS-related complex, and those with HIV infection but without AIDS symptoms. HIV infection is a transmissible disease ultimately characterized by a profound deficiency of cellular immune function as well as the

occurrence of opportunistic infection and malignancy. The primary immunologic abnormality resulting from infection by HIV is the progressive depletion and functional impairment of T lymphocytes expressing the CD4 cell surface glycoprotein (Lane *et al.*, *Ann. Rev. Immunol.*, 3:477 [1985]). The loss of CD4 helper/inducer T cell function probably underlies the profound defects in cellular and humoral immunity leading to the opportunistic infections and malignancies characteristic of AIDS (H. Lane *supra*).

Although the mechanism of HIV-associated ITP is unknown, it is believed to be different from the mechanism of ITP not associated with HIV infection. (Walsh *et al.*, *N. Eng. J. Med.*, 311:635-639 [1984]; and Ratner, *Am. J. Med.*, 86:194-198 [1989]).

IV. Current Therapy for Thrombocytopenia

The therapeutic approach to the treatment of patients with thrombocytopenia is dictated by the severity and urgency of the clinical situation. The treatment is similar for HIV-associated and non-HIV-related thrombocytopenia, and although a number of different therapeutic approaches have been used, the therapy remains controversial.

Platelet counts in patients diagnosed with thrombocytopenia have been successfully increased by glucocorticoid (*e.g.*, prednisolone) therapy, however in most patients, the response is incomplete, or relapse occurs when the glucocorticoid dose is reduced or its administration is discontinued. Based upon studies with patients having HIV-associated ITP, some investigators have suggested that glucocorticoid therapy may result in predisposition to AIDS. Glucocorticoids are usually administered if platelet count falls below 20 X 10^9/liter or when spontaneous bleeding occurs.

For patients refractory to glucocorticoids, the compound:

4-(2-chlorphenyl)-9-methyl-2-[3-(4-morpholinyl)-3-propanon-1-yl]6H-thieno[3,2,f][1,2,4]triazolo[4,3,a,][1,4]diazepin (WEB 2086)

has been successfully used to treat a severe case of non HIV-associated ITP. A patient having platelet counts of 37,000-58,000/µl was treated with WEB 2086 and after 1-2 weeks treatment platelet counts increased to 140,000-190,000/µl. (EP 361,077 and Lohman *et al.*, *Lancet*, 1147 [1988]).

Although the optimal treatment for acquired amegakaryocytic thrombocytopenia purpura (AATP) is uncertain, antithymocyte globulin (ATG), a horse antiserum to human thymus tissue, has been shown to produce prolonged complete remission (Trimble *et al.*, *Am. J. Hematol.*, 37:126-127 [1991]). A recent report however, indicates that the hematopoietic effects of ATG are attributable to thimerosal, where presumably the protein acts as a mercury carrier (Panella *et al.*, *Cancer Research*, 50:4429-4435 [1990]).

Good results have been reported with splenectomy. Splenectomy removes the major site of platelet destruction and a major source of autoantibody production in many patients. This procedure results in prolonged treatment-free remissions in a large number of patients. However, since surgical procedures are generally to be avoided in immune compromised patients, splenectomy is recommended only in severe cases of thrombocytopenia (*e.g.* severe HIV-associated ITP), in patients who fail to respond to 2 to 3 weeks of glucocorticoid treatment, or do not achieve sustained response after discontinuation of glucocorticoid administration. Based upon current scientific knowledge, it is unclear whether splenectomy predisposes patients to AIDS.

In addition to prednisolone therapy and splenectomy, certain cytotoxic agents, *e.g.*, vincristine, and azidothimidine (AZT, zidovudine) also show promise in treating HIV-induced ITP; however, the results are preliminary.

It will be appreciated from the foregoing that one way to treat thrombocytopenia would be to obtain an agent capable of accelerating the differentiation and maturation of megakaryocytes or precursors thereof into the platelet-producing form. Considerable efforts have been expended on identifying such an agent, commonly referred to as "thrombopoietin" (TPO). Other names for TPO commonly found in the literature include: thrombocytopoiesis stimulating factor (TSF), megakaryocyte colony-stimulating factor (MK-CSF), megakaryocyte-stimulating factor and megakaryocyte potentiator. TPO activity was observed as early as 1959 (Rak *et al., Med. Exp.*, 1:125) and attempts to characterize and purify this agent have continued to the present day. While reports of partial purification of TPO-active polypeptides exist (see, for example, Tayrien *et al., J. Biol. Chem.*, 262:3262 [1987] and Hoffman *et al., J. Clin. Invest.* 75:1174 [1985]), others have postulated that TPO is not a discrete entity in its own right but rather is simply the polyfunctional manifestation of a known hormone (IL-3, Sparrow *et al., Prog. Clin. Biol. Res.*, 215:123 [1986]). Regardless of its form or origin, a molecule possessing thrombopoietic activity would be of significant therapeutic value. Although no protein has been unambiguously identified as TPO, considerable interest surrounds the recent discovery that *mpl*, a putative cytokine receptor, may transduce a thrombopoietic signal.

V. *Mpl* is a Megakaryocytopoietic Cytokine Receptor

It is believed that the proliferation and maturation of hematopoietic cells is tightly regulated by factors that positively or negatively modulate pluripotential stem cell proliferation and multilineage differentiation. These effects are mediated through the high-affinity binding of extracellular protein factors to specific cell surface receptors. These cell surface receptors share considerable homology and are generally

classified as members of the cytokine receptor superfamily. Members of the superfamily include receptors for: IL-2 (β and γ chains) (Hatakeyama *et al.*, *Science*, **244**:551-556 [1989]; Takeshita *et al.*, *Science*, **257**:379-382 [1991]), IL-3 (Itoh *et al.*, *Science*, **247**:324-328 [1990]; Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, **87**:5459-5463 [1990]; Kitamura *et al.*, *Cell*, **66**:1165-1174 [1991a]; Kitamura *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**:5082-5086 [1991b]), IL-4 (Mosley *et al.*, *Cell*, **59**:335-348 [1989], IL-5 (Takaki *et al.*, *EMBO J.*, **9**:4367-4374 [1990]; Tavernier *et al.*, *Cell*, **66**:1175-1184 [1991]), IL-6 (Yamasaki *et al.*, *Science*, **241**:825-828 [1988]; Hibi *et al.*, *Cell*, **63**:1149-1157 [1990]), IL-7 (Goodwin *et al.*, *Cell*, **60**:941-951 [1990]), IL-9 (Renault *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**:5690-5694 [1992]), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Gearing *et al.*, *EMBO J.*, **8**:3667-3676 [1991]; Hayashida *et al.*, *Proc. Natl. Acad. Sci. USA*, **244**:9655-9659 [1990]), granulocyte colony-stimulating factor (G-CSF) (Fukunaga *et al.*, *Cell*, **61**:341-350 [1990a]; Fukunaga *et al.*, *Proc. Natl. Acad. Sci. USA*, **87**:8702-8706 [1990b]; Larsen *et al.*, *J. Exp. Med.*, **172**:1559-1570 [1990]), EPO (D'Andrea *et al.*, *Cell*, **57**:277-285 [1989]; Jones *et al.*, *Blood*, **76**:31-35 [1990]), Leukemia inhibitory factor (LIF) (Gearing *et al.*, *EMBO J.*, **10**:2839-2848 [1991]), oncostatin M (OSM) (Rose *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**:8641-8645 [1991]) and also receptors for prolactin (Boutin *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**:7744-7748 [1988]; Edery *et al.*, *Proc. Natl. Acad. Sci. USA*, **86**:2112-2116 [1989]), growth hormone (GH) (Leung *et al.*, *Nature*, **330**:537-543 [1987]) and ciliary neurotrophic factor (CNTF) (Davis *et al.*, *Science*, **253**:59-63 [1991].

Members of the cytokine receptor superfamily may be grouped into three functional categories (for review see Nicola *et al.*, *Cell*, **67**:1-4 [1991]). The first class comprises single chain receptors, such as erythropoietin receptor (EPO-R) or granulocyte colony stimulating factor receptor (G-CSF-R), which bind ligand with high affinity via the extracellular domain and also generate an intracellular signal. A second class of receptors, so called α-subunits, includes interleukin-6 receptor (IL6-R), granulocyte-macrophage colony stimulating factor receptor (GM-CSF-R), interleukin-3 receptor (IL3-Rα) and other members of the cytokine receptor superfamily. These α-subunits bind ligand with low affinity but cannot transduce an intracellular signal. A high affinity receptor capable of signaling is generated by a heterodimer between an α-subunit and a member of a third class of cytokine receptors, termed β-subunits, *e.g.*, β_C, the common β-subunit for the three α-subunits IL3-Rα and GM-CSF-R.

Evidence that *mpl* is a member of the cytokine receptor superfamily comes from sequence homology (Gearing, *EMBO J.*, **8**:3667-3676 [1988]; Bazan, *Proc.*

Natl. Acad. Sci. USA, 87:6834-6938 [1990]; Davis *et al., Science,* 253:59-63 [1991] and Vigon *et al., Proc. Natl. Acad. Sci. USA,* 89:5640-5644 [1992]) and its ability to transduce proliferative signals.

Deduced protein sequence from molecular cloning of murine c-*mpl* reveals this protein is homologous to other cytokine receptors. The extracellular domain contains 465 amino acid residues and is composed of two subdomains each with four highly conserved cysteines and a particular motif in the N-terminal subdomain and in the C-terminal subdomain. The ligand-binding extracellular domains are predicted to have similar double β-barrel fold structural geometries. This duplicated extracellular domain is highly homologous to the signal transducing chain common to IL-3, IL-5 and GM-CSF receptors as well as the low-affinity binding domain of LIF (Vigon *et al., Oncogene,* 8:2607-2615 [1993]). Thus *mpl* may belong to the low affinity ligand binding class of cytokine receptors

A comparison of murine *mpl* and mature human *mpl* P, reveals these two proteins show 81% sequence identity. More specifically, the N-terminus and C-terminus extracellular subdomains share 75% and 80% sequence identity respectively. The most conserved *mpl* region is the cytoplasmic domain showing 91% amino acid identity, with a sequence of 37 residues near the transmembrane domain being identical in both species. Accordingly, *mpl* is reported to be one of the most conserved members of the cytokine receptor superfamily (Vigon *supra*).

Evidence that *mpl* is a functional receptor capable of transducing a proliferative signal comes from construction of chimeric receptors containing an extracellular domain from a cytokine receptor having high affinity for a known cytokine with the *mpl* cytoplasmic domain. Since no known ligand for *mpl* has been reported, it was necessary to construct the chimeric high affinity ligand binding extracellular domain from a class one cytokine receptor such as IL-4R or G-CSFR Vigon *et al., supra* fused the extracellular domain of G-CSFR with both the transmembrane and cytoplasmic domain of c-*mpl*. An IL-3 dependent cell line, BAF/B03 (Ba/F3) was transfected with the G-CSFR/*mpl* chimera along with a full length G-CSFR control. Cells transfected with the chimera grew equally well in the presence of cytokine IL-3 or G-CSF. Similarly, cells transfected with G-CSFR also grew well in either IL-3 or G-CSF. All cells died in the absence of growth factors. A similar experiment was conducted by Skoda *et al., EMBO J.,* 12(7):2645-2653 [1993] in which both the extracellular and transmembrane domains of human IL-4 receptor (hIL-4-R) were fused to the murine *mpl* cytoplasmic domain, and transfected into a murine IL-3 dependent Ba/F3 cell line. Ba/F3 cells transfected with wild type hIL-4-R proliferated normally in the presence of either of the species specific IL-4 or IL-3. Ba/F3 cells transfected with hIL-4R/*mpl* proliferated

normally in the presence of hIL-4 (in the presence or absence of IL-3) demonstrating that in Ba/F3 cells the *mpl* cytoplasmic domain contains all the elements necessary to transduce a proliferative signal.

These chimeric experiments demonstrate the proliferation signaling capability of the *mpl* cytoplasmic domain but are silent regarding whether the *mpl* extracellular domain can bind a ligand. These results are consistent with at least two possibilities, namely, *mpl* is a single chain (class one) receptor like EPO-R or G-CSFR or it is a signal transducing β-subunit (class three) requiring an α-subunit like IL-3 (Skoda *et al. supra*).

VI. *Mpl* Ligand is a Thrombopoietin (TPO)

As described above, it has been suggested that serum contains a unique factor, sometimes referred to as thrombopoietin (TPO), that acts synergistically with various other cytokines to promote growth and maturation of megakaryocytes. No such natural factor has ever been isolated from serum or any other source even though considerable effort has been expended by numerous groups. Even though it is not known whether *mpl* is capable of directly binding a megakaryocyte stimulating factor, recent experiments demonstrate that *mpl* is involved in proliferative signal transduction from a factor or factors found in the serum of patients with aplastic bone marrow (Methia *et al.*, *Blood*, 82(5):1395-1401 [1993]).

Evidence that a unique serum colony-forming factor distinct from IL-1α, IL-3, IL-4, IL-6, IL-11, SCF, EPO, G-CSF, and GM-CSF transduces a proliferative signal through *mpl* comes from examination of the distribution of c-*mpl* expression in primitive and committed hematopoietic cell lines and from *mpl* antisense studies in one of these cell lines.

Using reverse transcriptase (RT)-PCR in immuno-purified human hematopoietic cells, Methia *et al., supra* demonstrated that strong *mpl* mRNA messages were only found in $CD34^+$ purified cells, megakaryocytes and platelets. $CD34^+$ cells purified from bone marrow (BM) represents about 1% of all BM cells and are enriched in primitive and committed progenitors of all lineages (*e.g.*, erythroid, granulomacrophage, and megakaryocytic).

Mpl antisense oligodeoxynucleotides were shown to suppress megakaryocytic colony formation from the pluripotent $CD34^+$ cells cultured in serum from patients with aplastic marrow (a rich source of megakaryocyte colony-stimulating activity [MK-CSA]). These same antisense oligodeoxynucleotides had no effect on erythroid or granulomacrophage colony formation.

Whether *mpl* directly bound a ligand and whether the serum factor shown to cause megakaryocytopoiesis acted through *mpl* was still unknown. It had been

suggested, however, that if *mpl* did directly bind a ligand, its amino acid sequence was likely to be highly conserved and have species cross-reactivity owing to the considerable sequence identity between human and murine *mpl* extracellular domains (Vigon *et al., supra* [1993]).

VII. Objects

In view of the foregoing, it will be appreciated there is a current and continuing need in the art to isolate and identify molecules capable of stimulating proliferation, differentiation and maturation of hematopoietic cells, especially megakaryocytes or their predecessors for therapeutic use in the treatment of thrombocytopenia. It is believed such a molecule is a *mpl* ligand and thus there exists a further need to isolate such ligand(s) to evaluate their role(s) in cell growth and differentiation.

Accordingly, it is an object of this invention to obtain a pharmaceutically pure molecule capable of stimulating proliferation, differentiation and/or maturation of megakaryocytes into the mature platelet-producing form.

It is another object to provide the molecule in a form for therapeutic use in the treatment of a hematopoietic disorder, especially thrombocytopenia

It is a further object of the present invention to isolate, purify and specifically identify protein ligands capable of binding *in vivo* a cytokine superfamily receptor known as *mpl* and to transduce a proliferative signal

It is still another object to provide nucleic acid molecules encoding such protein ligands and to use these nucleic acid molecules to produce *mpl* binding ligands in recombinant cell culture for diagnostic and therapeutic use

It is yet another object to provide derivatives and modified forms of the protein ligands including amino acid sequence variants, variant glycoprotein forms and covalent derivatives thereof.

It is an additional object to provide fusion polypeptide forms combining a *mpl* ligand and a heterologous protein and covalent derivatives thereof.

It is still an additional object to provide variant polypeptide forms combining a *mpl* ligand with amino acid additions and substitutions from the EPO sequence to produce a protein capable of regulating proliferation and growth of both platelets and red blood cell progenitors.

It is yet an additional object to prepare immunogens for raising antibodies against *mpl* ligands or fusion forms thereof, as well as to obtain antibodies capable of binding such ligands.

These and other objects of the invention will be apparent to the ordinary artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

The objects of the invention are achieved by providing an isolated mammalian megakaryocytopoietic proliferation and maturation promoting protein, denominated the "*mpl* ligand" (ML) or "thrombopoietin" (TPO), capable of stimulating proliferation, maturation and/or differentiation of megakaryocytes into the mature platelet-producing form.

This substantially homogeneous protein may be purified from a natural source by a method comprising; (1) contacting a source plasma containing the *mpl* ligand molecules to be purified with an immobilized receptor polypeptide, specifically *mpl* or a *mpl* fusion polypeptide immobilized on a support, under conditions whereby the *mpl* ligand molecules to be purified are selectively adsorbed onto the immobilized receptor polypeptide, (2) washing the immobilized receptor polypeptide and its support to remove non-adsorbed material, and (3) eluting the *mpl* ligand molecules from the immobilized receptor polypeptide to which they are adsorbed with an elution buffer. Preferably the natural source is mammalian plasma or urine containing the *mpl* ligand. Optionally the mammal is aplastic and the immobilized receptor is a *mpl*-IgG fusion.

Optionally, the prefered megakaryocytopoietic proliferation and maturation promoting protein is an isolated substantially homogeneous *mpl* ligand polypeptide made by synthetic or recombinant means

The "*mpl* ligand" polypeptide or "TPO" of this invention preferably has at least 70% overall sequence identity with the amino acid sequence of the highly purified substantially homogeneous porcine *mpl* ligand polypeptide and at least 80% sequence identity with the "EPO-domain" of the porcine *mpl* ligand polypeptide. Optionally, the *mpl* ligand of this invention is mature human *mpl* ligand (hML), having the mature amino acid sequence provided in **Fig. 1** (SEQ ID NO: 1), or a variant or posttranscriptionally modified form thereof or a protein having about 80% sequence identity with mature human *mpl* ligand. Optionally the *mpl* ligand variant is a fragment, especially an amino-terminus or "EPO-domain" fragment, of the mature human *mpl* ligand (hML). Preferably the amino terminus fragment retains substantially all of the human ML sequence between the first and forth cysteine residues but may contain substantial additions, deletions or substitutions outside that region. According to this embodiment, the fragment polypeptide may be represented by the formula:

X-hML(7-151)-Y

Where hML(7-151) represents the human TPO (hML) amino acid sequence from Cys^7 through Cys^{151} inclusive; X represents the amino group of Cys^7 or one or more of the

amino-terminus amino acid residue(s) of the mature hML or amino acid residue extensions thereto such as Met, Tyr or leader sequences containing, for example, proteolytic cleavage sites (*e.g.* Factor Xa or thrombin); and Y represents the carboxy terminal group of Cys^{151} or one or more carboxy-terminus amino acid residue(s) of the mature hML or extensions thereto.

Optionally the *mpl* ligand polypeptide or fragment thereof may be fused to a heterologous polypeptide (chimera). A preferred heterologous polypeptide is a cytokine, colony stimulating factor or interleukin or fragment thereof, especially kit-ligand (KL), IL-1, IL-3, IL-6, IL-11, EPO, GM-CSF or LIF. An optional preferred heterologous polypeptide is an immunoglobin chain, especially human IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgD, IgM or fragment thereof, especially comprising the constant domain of an IgG heavy chain.

Another aspect of this invention provides a composition comprising an isolated *mpl* agonist that is biologically active and is preferably capable of stimulating the incorporation of labeled nucleotides (*e.g.*, ^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl*. Optionally the *mpl* agonist is biologically active *mpl* ligand and is preferably capable of stimulating the incorporation of ^{35}S into circulating platelets in a mouse platelet rebound assay. Suitable *mpl* agonist include hML_{153}, hML(R153A, R154A), hML2, hML3, hML4, mML, mML2, mML3, pML, and pML2 or fragments thereof.

In another embodiment, this invention provides an isolated antibody capable of binding to the *mpl* ligand. The isolated antibody capable of binding to the *mpl* ligand may optionally be fused to a second polypeptide and the antibody or fusion thereof may be used to isolate and purify *mpl* ligand from a source as described above for immobilized *mpl*. In a further aspect of this embodiment, the invention provides a method for detecting the *mpl* ligand *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the ligand and detecting if binding has occurred.

In still further embodiments, the invention provides an isolated nucleic acid molecule, encoding the *mpl* ligand or fragments thereof, which nucleic acid molecule may optionally be labeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under moderate to highly stringent conditions with, a nucleic acid molecule having a sequence encoding a *mpl* ligand. Preferred nucleic acid molecules are those encoding human, porcine, and murine *mpl* ligand, and include RNA and DNA, both genomic and cDNA. In a further aspect of this embodiment, the nucleic acid molecule is DNA encoding the *mpl* ligand and further comprises a replicable vector in which the DNA is operably linked to control sequences

recognized by a host transformed with the vector. Optionally the DNA is cDNA having the sequence provided in **Fig. 1** 5'-3' (SEQ ID NO: 2), 3'-5' or a fragment thereof. This aspect further includes host cells, preferably CHO cells, transformed with the vector and a method of using the DNA to effect production of *mpl* ligand, preferably comprising expressing the cDNA encoding the *mpl* ligand in a culture of the transformed host cells and recovering the *mpl* ligand from the host cells or the host cell culture. The *mpl* ligand prepared in this manner is preferably human *mpl* ligand.

The invention further includes a method for treating a mammal having a hematopoietic disorder, especially thrombocytopenia, comprising administering a therapeutically effective amount of a *mpl* ligand to the mammal. Optionally the *mpl* ligand is administered in combination with a cytokine, especially a colony stimulating factor or interleukin. Preferred colony stimulating factors or interleukins include; kit-ligand (KL), LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-3, IL-6, and IL-11.

The invention further includes a process for isolating and purifying TPO (ML) from a TPO producing microorganism comprising.

(1) disrupting or lysing cells containing TPO.

(2) optionally seperating soluble material from insoluble material containing TPO.

(3) solublizing TPO in the insoluble material with a solublizing buffer.

(4) seperating solublized TPO from other soluble and insoluble material.

(5) refolding TPO in a redox buffer, and

(6) separating properly folded TPO from misfolded TPO.

The process provides for solubilizing the insoluble material containing TPO with a chaotropic agent where the chaotropic agent is selected from a salt of guanidine, sodium thiocyanate, or urea. The process further provides that solublized TPO is seperated from other soluble and insoluble material by one or more steps selected from centrafugation, gel filtration and reverse phase chromotography. The refolding step of the process provides for a redox buffer containing both an oxidizing and reducing agent. Generally, the oxidizing agent is oxygen or a compound containing at least one disulfide bond and the reducing agent is a compound containing at least one free sulfhydryl. Preferably, the oxidizing agent is selected from oxidized glutathione(GSSG) and cystine and the reducing agent is selected from reduced glutathione(GSH) and cysteine. Most preferably the oxidizing agent is oxidized glutathione(GSSG) and the reducing agent is reduced glutathione(GSH). It is also prefered that the molar ratio of the oxidizing agent is equal to or greater then that of the reducing agent. The redox buffer additionally contains a detergent, preferably selected from CHAPS and CHAPSO, present at a level of at least1%. The redox buffer additionally contains NaCl preferably at a concentration range of about 0.1-0.5M, and

glycerol preferably at a concentration greater than 15%. The pH of the redox buffer preferably ranges from about pH 7.5-pH 9.0. and the refolding step is conducted at 4 degrees for 12-48hr. The refolding step produces biologically active TPO in which a disulfide bond is formed between the Cys nearest the amino-terminus with the Cys nearest the carboxy-terminus of the EPO domain.

The invention further includes a process for purifying biologically active TPO from a microorganism comprising.

(1) lysing at least the extracellular membrane of the microorganism,

(2) treating the lysate containing TPO with a chaotropic agent,

(3) refolding the TPO, and

(4) separating impurities and misfolded TPO from properly folded TPO.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the deduced amino acid sequence (SEQ ID NO: 1) of human *mpl* ligand (hML) cDNA and the coding nucleotide sequence (SEQ ID NO: 2). Nucleotides are numbered at the beginning of each line The 5' and 3' untranslated regions are indicated in lower case letters. Amino acid residues are numbered above the sequence starting at Ser 1 of the mature *mpl* ligand (ML) protein sequence. The boundries of presumed exon 3 are indicated by the arrows and the potential N-glycosylation sites are boxed. Cysteine residues are indicated by a dot above the sequence. The underlined sequence corresponds to the N-terminal sequence determined from *mpl* ligand purified from porcine plasma.

Fig. 2 shows the procedure used for the *mpl* ligand ^{3}H-thymidine incorporation assay. To determine the presence of *mpl* ligand from various sources, the *mpl* P Ba/F3 cells were starved of IL-3 for 24 hours in a humidified incubator at 37°C in 5% CO_2 and air. Following IL-3 starvation the cells were plated out in 96 well culture dishes with or without diluted samples and cultured for 24 hrs in a cell culture incubator. 20 µl of serum free RPMI media containing 1 µCi of ^{3}H-thymidine was added to each well for the last 6-8 hours. The cells were then harvested on 96 well filter plates and washed with water. The filters were then counted.

Fig. 3 shows the effect of pronase, DTT and heat on the ability of APP to stimulate Ba/F3-*mpl* cell proliferation. For pronase digestion of APP, pronase (Boehringer Mannheim) or bovine serum albumin was coupled to Affi-gel10 (Biorad) and incubated individually with APP for 18hrs. at 37°C. Subsequently, the resins were

removed by centrifugation and supernatants assayed. APP was also heated to 80°C for 4 min. or made 100 μM DTT followed by dialysis against PBS.

Fig. 4 shows the elution of *mpl* ligand activity from Phenyl-Toyopearl, Blue-Sepharose and Ultralink-*mpl* columns. Fractions 4-8 from the *mpl* affinity column were the peak activity fractions eluted from the column.

Fig. 5 shows the SDS-PAGE of eluted Ultralink-*mpl* fractions. To 200 μl of each fraction 2-8, 1 ml of acetone containing 1mM HCl at -20°C was added. After 3hrs. at -20°C samples were centrifuged and resultant pellets were washed 2x with acetone at -20°C. The acetone pellets were subsequently dissolved in 30 μl of SDS-solubilization buffer, made 100 μM DTT and heated at 90°C for 5 min. The samples were then resolved on a 4-20% SDS-polyacrylamide gel and proteins were visualized by silver staining.

Fig. 6 shows elution of *mpl* ligand activity from SDS-PAGE. Fraction 6 from the *mpl*-affinity column was resolved on a 4-20% SDS-polyacrylamide gel under non-reducing conditions. Following electrophoresis the gel was sliced into 12 equal regions and electroeluted as described in the examples. The electroeluted samples were dialyzed into PBS and assayed at a 1/20 dilution. The Mr standards used to calibrate the gel were Novex Mark 12 standards.

Fig. 7 shows the effect of *mpl* ligand depleted APP on human megakaryocytopoiesis. *mpl* ligand depleted APP was made by passing 1 ml over a 1 ml *mpl*-affinity column (700 μg *mpl*-IgG/ml NHS-superose, Pharmacia). Human peripheral stem cell cultures were made 10% APP or 10% *mpl* ligand depleted APP and cultured for 12 days. Megakaryocytopoiesis was quantitated as described in the examples

Fig. 8 shows the effect of *mpl*-IgG on the stimulation of human megakaryocytopoiesis by APP. Human peripheral stem cell cultures were made 10% with APP and cultured for 12 days. At day 0, 2 and 4, *mpl*-IgG (0.5 μg) or ANP-R-IgG (0.5 μg) was added. After 12 days megakaryocytopoiesis was quantitated as described in the examples. The average of duplicate samples is graphed with the actual duplicate data in parenthesis.

Fig. 9 shows both strands of a 390 bp fragment of human genomic DNA encoding the *mpl* ligand. The deduced amino acid sequence of "exon 3" (SEQ ID NO: 3), the coding sequence (SEQ ID NO: 4), and its compliment (SEQ ID NO: 5) are shown.

Fig. 10 shows deduced amino acid sequence of mature human *mpl* ligand (hML) (SEQ ID NO: 6) and mature human erythropoietin (hEPO) (SEQ ID NO: 7). The predicted amino acid sequence for the human *mpl* ligand is aligned with the human erythropoietin sequence. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Potential N-glycosylation sites are underlined with a plain line for the hML and with a broken line for hEPO. The two cysteines important for erythropoietin activity are indicated by a large dot.

Fig. 11 shows deduced amino acid sequence of mature human *mpl* ligand isoforms hML (SEQ ID NO: 6), hML2 (SEQ ID NO: 8), hML3 (SEQ ID NO: 9), and hML4 (SEQ ID NO: 10). Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes.

Figs. 12A, 12B and 12C show the effect of human *mpl* ligand on Ba/F3-*mpl* cell proliferation **(A)**, *in vitro* human megakaryocytopoiesis quantitated using a radiolabeled murine IgG monoclonal antibody specific to the megakaryocyte glycoprotein $GPII_bIII_a$ **(B)**, and murine thrombopoiesis measured in a platelet rebound assay **(C)**.

Two hundred ninety-three cells were transfected by the $CaPO_4$ method (Gorman, C in *DNA Cloning : A New Approach* **2**:143-190 [1985]) with pRK5 vector alone, pRK5-hML or with pRK5-ML_{153} overnight (pRK5-ML_{153} was generated by introducing a stop codon after residue 153 of hML by PCR). Media was then conditioned for 36h and assayed for stimulation of cell proliferation of Ba/F3-*mpl* as described in **Example 1** **(A)** or *in vitro* human megakaryocytopoiesis **(B)**. Megakaryocytopoiesis was quantitated using a ^{125}I radiolabeled murine IgG monoclonal antibody (HP1-1D) to the megakaryocyte specific glycoprotein $GPII_bIII_a$ as described (Grant *et al., Blood* **69**:1334-1339 [1987]). The effect of partially purified recombinant ML (rML) on *in vivo* platelet production **(C)** was determined using the rebound thrombocytosis assay described by McDonald, T.P. *Proc. Soc. Exp. Biol. Med.* **144**:1006-10012 (1973). Partially purified rML was prepared from 200ml of conditioned media containing the recombinant ML. The media was passed through a 2ml Blue-Separose column equilabrated in PBS and the column was washed with PBS and eluted with PBS containing 2M each of urea and NaCl. The active fraction was dialyzed into PBS and made 1mg/ml with endotoxin free BSA. The sample contained less than one unit of endotoxin /ml. Mice were injected with either 64,000, 32,000 or 16,000 units of rML or excipient alone. Each group consisted of six mice The mean and standard deviation of each group is shown. p values were determined by a 2 tailed T-test comparing medians.

Fig. 13 compares the effect of human *mpl* ligand isoforms and variants in the Ba/F3-*mpl* cell proliferation assay. hML, mock, hML2, hML3, hML(R153A, R154A), and hML_{153} were assayed at various dilutions as described in **Example 1.**

Figs. 14A, 14B and **14C** show the deduced amino acid sequence (SEQ ID NO: 1) of human *mpl* ligand (hML) or human TPO (hTPO) and the human genomic DNA coding sequence (SEQ ID NO: 11). Nucleotides and amino acid residues are numbered at the beginning of each line.

Fig. 15 shows a SDS-PAGE of purified $293\text{-}rhML_{332}$ and purified $293\text{-}rhML_{153}$.

Fig. 16 shows the nucleotide sequence: cDNA coding (SEQ ID NO: 12) and deduced amino acid sequence (SEQ ID NO: 13) of the open reading frame of a murine ML isoform. This mature murine *mpl* ligand isoform contains 331 amino acid residues, four fewer than the putative full length mML, and is therefore designated mML2. Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1. The potential N-glycosylation sites are underlined Cysteine residues are indicated by a dot above the sequence.

Fig. 17 shows the cDNA sequence (SEQ ID NO: 14) and predicted protein sequence (SEQ ID NO: 15) of this murine ML isoform (mML). Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1. This mature murine *mpl* ligand isoform contains 335 amino acid residues and is believed to be the full length *mpl* ligand, designated mML. The signal sequence is indicated with a dashed underline and the likely cleavage point is denoted with an arrow. The 5' and 3' untranslated regions are indicated with lower case letters. The two deleations found as a result of alternative splicing (mML2 and mML3) are underlined. The four cysteine residues are indicated by a dot. The seven potential N-glycosylation sites are boxed.

Fig. 18 compares the deduced amino acid sequence of the human ML isoform hML3 (SEQ ID NO: 9) and a murine ML isoform designated mML3 (SEQ ID NO: 16). The predicted amino acid sequence for the human *mpl* ligand is aligned with the murine *mpl* ligand sequence. Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Amino acids are numbered at the beginning of each line.

Fig. 19 compares the predicted amino acid sequences of mature ML isoforms from mouse-ML (SEQ ID NO: 17), porcine-ML (SEQ ID NO: 18) and human-ML (SEQ ID NO: 6). Amino acid sequences are aligned with gaps, indicated by dashes, introduced for optimal alingment. Amino acids are numbered at the beginning of each line with identical residues boxed. Potential N-glycosylation sites are indicated by a shaded box and cysteine residues are designated with a dot. The conserved di-basic amino acid motif that presents a potential protease cleavage site is underlined. The four amino acid deletion found to occur in all three species (ML2) is outlined with a bold box.

Fig. 20 shows the cDNA sequence (SEQ ID NO: 19) and predicted mature protein sequence (SEQ ID NO: 18) of a porcine ML isoform (pML). This porcine *mpl* ligand isoform contains 332 amino acid residues and is believed to be the full length porcine *mpl* ligand, designated pML. Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1.

Fig. 21 shows the cDNA sequence (SEQ ID NO: 20) and predicted mature protein sequence (SEQ ID NO: 21) of a porcine ML isoform (pML2). This porcine *mpl* ligand isoform contains 328 amino acid residues and is a four residues deletion form of the full length porcine *mpl* ligand, designated pML2 Nucleotides are numbered at the beginning of each line. Amino acid residues are numbered above the sequence starting with Ser 1.

Fig. 22 compares the deduced amino acid sequence of the full length porcine ML isoform pML (SEQ ID NO: 18) and a porcine ML isoform designated pML2 (SEQ ID NO: 21). The predicted amino acid sequence for the pML is aligned with pML2 sequence Identical amino acids are boxed and gaps introduced for optimal alignment are indicated by dashes. Amino acids are numbered at the beginning of each line.

Fig. 23 shows the pertinent features of plasmid pSVI5.ID.LL.MLORF ("full length" or TPO_{332}) used to transfect host CHO-DP12 cells for production of CHO-rhTPO_{332}.

Fig. 24 shows the pertinent features of plasmid pSVI5.ID.LL.MLEPO-D ("truncated" or TPO_{153}) used to transfect host CHO-DP12 cells for production of CHO-rhTPO_{153}.

Figs. 25A, 25B, and 25C show the effect of *E. coli*-rhTPO(Met^{-1}, 153) on platelets (A), red blood cells (B) and (C) white blood cells in normal mice. Two groups of 6 female C57 B6 mice were injected daily with either PBS buffer or 0.3μg *E. coli*-rhTPO(Met^{-1}, 153) (100μl sc.). On day 0 and on days 3-7 40μl of blood was

taken from the orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018. The data are presented as means ± Standard error of the mean.

Figs. 26A, 26B and **26C** show the effect of *E. coli*-rhTPO(Met^{-1}, 153) on platelets (**A**), red blood cells (**B**) and (**C**) white blood cells in sublethally irradiated mice. Two groups of 10 female C57 B6 mice were sublethally irradiated with 750 cGy of gamma radiation from a ^{137}Cs source and injected daily with either PBS buffer or 3.0µg *E. coli*-rhTPO(Met^{-1}, 153) (100µl sc.). On day 0 and at subsequent intermediate time points 40µl of blood was taken from the orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018. The data are presented as means ± Standard error of the mean.

Figs. 27A. 27B and **27C** show the effect of CHO-rhTPO$_{332}$ on (**A**) platelets (thrombocytes), (**B**) red blood cells (erythrocytes) and (**C**) white blood cells (leukocytes) in normal mice. Two groups of 6 female C57 B6 mice were injected daily with either PBS buffer or 0.3µg CHO-rhTPO$_{332}$ (100µl sc.). On day 0 and on days 3-7 40µl of blood was taken from the orbital sinus. This blood was immediately diluted in 10 ml of commercial diluant and complete blood counts were obtained on a Serrono Baker Hematology Analyzer 9018 The data are presented as means ± Standard error of the mean.

Fig. 28 shows dose response curves for various forms of rhTPO obtained from various cell lines. Dose response curves were constructed to rhTPO from the following cell lines: hTPO$_{332}$ from CHO (full length from Chinese hamster ovary cells); hTPO$_{Met^{-1}}$ $_{153}$ (E. coli-derived truncated form with an N-terminal methionine from); hTPO$_{332}$ (full length TPO from human 293 cells); Met-less 155 E-Coli (the truncated form [rhTPO$_{155}$] without the terminal methionine from E coli). Groups of 6 female C57B6 mice were injected daily for 7 days with rhTPO depending upon group. Each day 40µl of blood was taken from the orbital sinus for complete blood counts. The data presented above are the maximal effects seen with the various treatments and with the exception of (met 153 *E-Coli*) this occurred on day 7 of treatment. In the aforementioned "met 153 *E-Coli*" group the maximal effect was seen on day 5. The data are presented as means ± Standard error of the mean.

Fig. 29 shows dose response curves comparing the activity of full length and "cliped" forms of rhTPO produced in CHO cells with the truncated form from *E coli.* Groups of 6 female C57B6 mice were injected daily with 0.3μg rhTPO of various types. On days 2-7 40μl of blood was taken from the orbital sinus for complete blood counts. Treatment groups were TPO_{153} the truncated form of TPO from *E coli*; TPO_{332} (Mix fraction) Full length TPO containing approximately 80-90% full length and 10-20% clipped forms; TPO332(30K fraction) = purified clipped fraction from the original "mix" preparation; TPO332(70K fraction) = purified full length TPO fraction from the original "mix" preparation. The data are presented as means $\pm$ Standard error of the mean.

Fig. 30 is a cartoon showing the KIRA ELISA assay for measuring TPO. The figure shows the MPL/Rse.gD chimera and relavant parts of the parent receptors as well as the final construct (right portion of the figure) and a flow diagram (left portion of the figure) showing relavant steps of the assay

Fig. 31 is a flow chart for the KIRA ELISA assay showing each step in the procedure.

Figs. 32A-32L provide the nucleotide sequence (SEQ ID NO: 22) of the pSVI17.ID.LL expression vector used for expression of Rse.gD in **Example 17**.

Fig. 33 is a schematic representation of the preperation of plasmid pMP1.

Fig. 34 is a schematic representation of the preperation of plasmid pMP21.

Fig. 35 is a schematic representation of the preperation of plasmid pMP151.

Fig. 36 is a schematic representation of the preperation of plasmid pMP202.

Fig. 37 is a schematic representation of the preperation of plasmid pMP172.

Fig. 38 is a schematic representation of the preperation of plasmid pMP210.

Fig. 39 is a table of the five best expressing TPO clones from the pMP210 plasmid bank (SEQ ID NOS: 23, 24, 25, 26, 27 and 28)

Fig. 40 is a schematic representation of the preperation of plasmid pMP41.

Fig. 41 is a schematic representation of the preperation of plasmid pMP57.

Fig. 42 is a schematic representation of the preperation of plasmid pMP251.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

"Chaotropic agent" refers to a compound which, in aqueous solution and in suitable concentrations, can cause a change in the spatial configuration or conformation of a protein by at least partially disrupting the forces responsible for maintaining the normal secondary and tertiary structure of the protein. Such compounds include, for example, urea, guanidine·HCl, and sodium thiocyanate. High concentrations, usually 4-9M, of these compounds are normally required to exert the conformational effect on proteins.

"Cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone, insulin-like growth factors, human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prorelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and leutinizing hormone (LH), hematopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor-α (TNF-α and TNF-β) mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, nerve growth factors such as NGF-β, platelet-growth factor, transforming growth factors (TGFs) such as TGF-α and TGF-β, insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons such as interferon-α, -β, and -γ, colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF), interleukins (IL's) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 and other polypeptide factors including LIF, SCF, and kit-ligand. As used herein the foregoing terms are meant to include proteins from natural sources or from recombinant cell culture. Similarly, the terms are intended to include biologically active equivalents; *e.g.*, differing in amino acid sequence by one or more amino acids or in type or extent of glycosylation.

"*mpl* ligand", "*mpl* ligand polypeptide", "ML", "thrombopoietin" or "TPO" are used interchangeably herein and comprise any polypeptide that possesses the property of binding to *mpl*, a member of the cytokine receptor superfamily, and having a biological property of the ML as defined below. An exemplary biological property is the ability to stimulate the incorporation of labeled nucleotides (*e.g.*, ^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P. Another exemplary biological property is the ability to stimulate the incorporation of ^{35}S into circulating platelets in a mouse platelet rebound assay. This definition encompasses the polypeptide isolated from a *mpl* ligand source such as aplastic porcine plasma described herein or from another source, such as another animal species, including humans or prepared by recombinant or synthetic methods and includes variant forms including functional derivatives, fragments, alleles, isoforms and analogues thereof.

A "*mpl* ligand fragment" or "TPO fragment" is a portion of a naturally occurring mature full length *mpl* ligand or TPO sequence having one or more amino acid residues or carbohydrate units deleted. The deleted amino acid residue(s) may occur anywhere in the peptide including at either the N-terminal or C-terminal end or internally. The fragment will share at least one biological property in common with *mpl* ligand. *Mpl* ligand fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of the *mpl* ligand isolated from a mammal including the ligand isolated from aplastic porcine plasma or the human or murine ligand, especially the EPO-domain thereof. Representative examples of N-terminal fragments are hML_{153} or TPO(Met^{-1}1-153).

"*Mpl* ligand variants" or "*mpl* ligand sequence variants" as defined herein means a biologically active *mpl* ligand as defined below having less than 100% sequence identity with the *mpl* ligand isolated from recombinant cell culture or aplastic porcine plasma or the human ligand having the deduced sequence described in **Fig. 1** (SEQ ID NO: 1). Ordinarily, a biologically active *mpl* ligand variant will have an amino acid sequence having at least about 70% amino acid sequence identity with the *mpl* ligand isolated from aplastic porcine plasma or the mature murine or human ligand or fragments thereof (see **Fig. 1** [SEQ ID NO: 1]), preferably at least about 75%, more preferably at least about 80%, still more preferably at least about 85%, even more preferably at least about 90%, and most preferably at least about 95%.

A "chimeric *mpl* ligand" is a polypeptide comprising full length *mpl* ligand or one or more fragments thereof fused or bonded to a second heterologous polypeptide or one or more fragments thereof. The chimera will share at least one biological property in common with *mpl* ligand. The second polypeptide will typically be a cytokine, immunoglobin or fragment thereof.

"Isolated *mpl* ligand", "highly purified *mpl* ligand" and "substantially homogeneous *mpl* ligand" are used interchangeably and mean a *mpl* ligand that has been purified from a *mpl* ligand source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means less than about 5% contamination with other source proteins.

"Biological property" when used in conjunction with either the "*mpl* ligand" or "Isolated *mpl* ligand" means having thrombopoietic activity or having an *in vivo* effector or antigenic function or activity that is directly or indirectly caused or performed by a *mpl* ligand (whether in its native or denatured conformation) or a fragment thereof. Effector functions include*mpl* binding and any carrier binding activity, agonism or antagonism of *mpl*, especially transduction of a proliferative signal including replication, DNA regulatory function, modulation of the biological activity of other cytokines, receptor (especially cytokine) activation, deactivation, up- or down regulation, cell growth or differentiation and the like. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the native *mpl* ligand. The principal antigenic function of a *mpl* ligand polypeptide is that it binds with an affinity of at least about 10^6 l/mole to an antibody raised against the *mpl* ligand isolated from aplastic porcine plasma. Ordinarily, the polypeptide binds with an affinity of at least about 10^7 l/mole. Most preferably, the antigenically active *mpl* ligand polypeptide is a polypeptide that binds to an antibody raised against the *mpl* ligand having one of the above described effector functions. The antibodies used to define "biologically activity" are rabbit polyclonal antibodies raised by formulating the *mpl* ligand isolated from recombinant cell culture or aplastic porcine plasma in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of *mpl* ligand antibody plateaus.

"Biologically active" when used in conjunction with either the "*mpl* ligand" or "Isolated *mpl* ligand" means a *mpl* ligand or polypeptide that exhibits thrombopoietic activity or shares an effector function of the *mpl* ligand isolated from aplastic porcine plasma or expressed in recombinant cell culture described herein. A principal known effector function of the *mpl* ligand or polypeptide herein is binding to *mpl* and stimulating the incorporation of labeled nucleotides (^{3}H-thymidine) into the DNA of

IL-3 dependent Ba/F3 cells transfected with human *mpl* P. Another known effector function of the *mpl* ligand or polypeptide herein is the ability to stimulate the incorporation of ^{35}S into circulating platelets in a mouse platelet rebound assay. Yet another known effector function of *mpl* ligand is the ability to stimulate *in vitro* human megakaryocytopoiesis that may be quantitated by using a radio labeled monoclonal antibody specific to the megakaryocyte glycoprotein $GPII_bIII_a$.

"Percent amino acid sequence identity" with respect to the *mpl* ligand sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the *mpl* ligand sequence isolated from aplastic porcine plasma or the murine or human ligand having the deduced amino acid sequence described in Fig. 1 (SEQ ID NO: 1), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the *mpl* ligand sequence shall be construed as affecting sequence identity or homology. Thus exemplary biologically active *mpl* ligand polypeptides considered to have identical sequences include; prepro-*mpl* ligand, pro-*mpl* ligand, and mature *mpl* ligand.

"*Mpl* ligand microsequencing" may be accomplished by any appropriate standard procedure provided the procedure is sensitive enough. In one such method, highly purified polypeptide obtained from SDS gels or from a final HPLC step are sequenced directly by automated Edman (phenyl isothiocyanate) degradation using a model 470A Applied Biosystems gas phase sequencer equipped with a 120A phenylthiohydantion (PTH) amino acid analyzer. Additionally, *mpl* ligand fragments prepared by chemical (*e.g.*, CNBr, hydroxylamine, 2-nitro-5-thiocyanobenzoate) or enzymatic (*e.g.*, trypsin, clostripain, staphylococcal protease) digestion followed by fragment purification (*e.g.*, HPLC) may be similarly sequenced PTH amino acids are analyzed using the ChromPerfect data system (Justice Innovations, Palo Alto, CA) Sequence interpretation is performed on a VAX 11/785 Digital Equipment Co. computer as described by Henzel *et al.*, *J. Chromatography*, 404:41-52 [1987]. Optionally, aliquots of HPLC fractions may be electrophoresed on 5-20% SDS-PAGE, electrotransferred to a PVDF membrane (ProBlott, AIB, Foster City, CA) and stained with Coomassie Brilliant Blue (Matsurdiara, *J. Biol. Chem.*, 262:10035-10038 [1987]. A specific protein identified by the stain is excised from the blot and N-terminal sequencing is carried out with the gas phase sequenator described above. For internal protein sequences, HPLC fractions are dried under vacuum (SpeedVac), resuspended in appropriate buffers, and digested with cyanogen bromide, the Lys-specific enzyme Lys-C (Wako Chemicals, Richmond, VA), or Asp-N (Boehringer Mannheim, Indianapolis, IN). After digestion, the resultant peptides are sequenced as a

mixture or after HPLC resolution on a C4 column developed with a propanol gradient in 0.1% TFA prior to gas phase sequencing

"Thrombocytopenia" is defined as a platelet count below 150 X 10^9 per liter of blood.

"Thrombopoietic activity" is defined as biological activity that consists of accelerating the proliferation, differentiation and/or maturation of megakaryocytes or megakaryocyte precursors into the platelet producing form of these cells. This activity may be measured in various assays including an *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-$GPII_bIII_a$) for a human leukemia megakaryoblastic cell line (CMK), and induction of polyploidization in a megakaryoblastic cell line (DAMI)

"Thrombopoietin" (TPO) is defined as a compound having thrombopoietic activity or being capable of increasing serum platelet counts in a mammal. TPO is preferably capable of increasing endogenous platelet counts by at least 10%, more preferably by 50%, and most preferably capable of elevating platelet counts in a human to greater that 150X10^9 per liter of blood.

"Isolated *mpl* ligand nucleic acid" is RNA or DNA containing greater than 16 and preferably 20 or more sequential nucleotide bases that encode biologically active *mpl* ligand or a fragment thereof, is complementary to the RNA or DNA, or hybridizes to the RNA or DNA and remains stably bound under moderate to stringent conditions This RNA or DNA is free from at least one contaminating source nucleic acid with which it is normally associated in the natural source and preferably substantially free of any other mammalian RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is present in the source or natural cell but is in a different chromosomal location or is otherwise flanked by nucleic acid sequences not normally found in the source cell An example of isolated *mpl* ligand nucleic acid is RNA or DNA that encodes a biologically active *mpl* ligand sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the human, murine or porcine *mpl* ligand.

"Control sequences" when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice

"Exogenous" when referring to an element means a nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

"Cell," "cell line," and "cell culture" are used interchangeably herein and such designations include all progeny of a cell or cell line. Thus, for example, terms like "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended it will be clear from the context.

"Plasmids" are autonomously replicating circular DNA molecules possessing independent origins of replication and are designated herein by a lower case "p" preceded and/or followed by capital letters and/or numbers The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from such available plasmids in accordance with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" when referring to DNA means catalytic cleavage of internal phosphodiester bonds of DNA with an enzyme that acts only at certain locations or sites in the DNA sequence. Such enzymes are called "restriction endonucleases". Each restriction endonuclease recognizes a specific DNA sequence called a "restriction site" that exhibits two-fold symmetry. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital

letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in sections 1.56-1.61 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* [New York: Cold Spring Harbor Laboratory Press, 1989].

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, *Nucleic Acids Res.*, 9:6103-6114 [1981], and Goeddel *et al.*, *Nucleic Acids Res.*, 8:4057 [1980].

"Southern analysis" or "Southern blotting" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook *et al.*, *supra*.

"Northern analysis" or "Northern blotting" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe,

using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

"Ligation" is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 μg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large- and small-scale plasmid preparations described in sections 1.25-1.33 of Sambrook *et al.*, *supra*. After preparation of the DNA it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid-phase techniques such as described in EP 266,032 published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, *Nucl. Acids Res.*, 14:5399-5407 [1986]). Further methods include the polymerase chain reaction defined below and other autoprimer methods and oligonucleotide syntheses on solid supports. All of these methods are described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, 28:716-734 (1989). These methods are used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides are then purified on polyacrylamide gels.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, *etc.* See generally Mullis *et al., Cold Spring Harbor Symp. Quant. Biol.,* 51:263 [1987]; Erlich. ed., *PCR Technology,* (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% $NaDodSO_4$ (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

"Moderately stringent conditions" are described in Sambrook *et al., supra* and include the use of a washing solution and hybridization conditions (*e.g.*, temperature, ionic strength, and %SDS) less stringent than described above. An example of moderately stringent conditions are conditions such as overnight incubation at 37°C in a solution comprising: 20% formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10% dextran sulfate, and 20 μl/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 X SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength *etc.* as necessary to accommodate factors such as probe length and the like.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like

molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one and (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, *J. Mol. Biol.*, **186**:651-663 [1985]; Novotny and Haber, *Proc. Natl Acad Sci. USA*, **82**:4592-4596 [1985]).

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, National Institute of Health, Bethesda, MD [1987]). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen binding fragments, called "Fab" fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda (λ), based on the amino acid sequences of their constant domains

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each

monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567 [Cabilly *et al.*]).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567 (Cabilly *et al.*); and Morrison *et al.*, *Proc. Natl. Acad Sci. USA*, 81:6851-6855 [1984])

"Humanized" forms of non-human (*e g.*. murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones

et al., *Nature*, 321:522-525 [1986]; Reichmann *et al.*, *Nature*, 332:323-329 [1988]; and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 [1992]).

"Non-immunogenic in a human" means that upon contacting the polypeptide in a pharmaceutically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide is demonstratable upon the second administration of the polypeptide after an appropriate latent period (*e.g.*, 8 to 14 days).

II. Preferred Embodiments of the Invention

Preferred polypeptides of this invention are substantially homogeneous polypeptide(s), referred to as *mpl* ligand(s) or thrombopoietin (TPO), that possesse the property of binding to *mpl*, a member of the receptor cytokine superfamily, and having the biological property of stimulating the incorporation of labeled nucleotides (^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P. More preferred *mpl* ligand(s) are isolated mammalian protein(s) having hematopoietic, especially megakaryocytopoietic or thrombocytopoietic activity - namely, being capable of stimulating proliferation, maturation and/or differentiation of immature megakaryocytes or their predecessors into the mature platelet-producing form. Most preferred polypeptides of this invention are human *mpl* ligand(s) including fragments thereof having hematopoietic, megakaryocytopoietic or thrombopoietic activity. Optionally these human *mpl* ligand(s) lack glycosylation. Other prefered human *mpl* ligands are the "EPO-domain" of hML refered to as hML_{153} or $hTPO_{153}$, a truncated form of hML refered to as hML_{245} or $hTPO_{245}$ and the mature full length polypeptide having the amino acid sequence shown in **Fig. 1** (SEQ ID NO: 1), refered to as hML, hML_{332} or $hTPO_{332}$ and the biolocically active substitutional variant hML(R153A, R154A).

Optional preferred polypeptides of this invention are biologically or immunologically active *mpl* ligands variants selected from hML2, hML3, hML4, mML, mML2, mML3, pML and pML2.

Optional preferred polypeptides of this invention are biologically active *mpl* ligand variant(s) that have an amino acid sequence having at least 70% amino acid sequence identity with the human *mpl* ligand (see **Fig. 1** [SEQ ID NO: 1]), the murine *mpl* ligand (see **Fig. 16** [SEQ ID NOS: 12 & 13]), the recombinant porcine *mpl* ligand (see **Fig. 19** [SEQ ID NO: 18]) or the porcine *mpl* ligand isolated from aplastic porcine plasma, preferably at least 75%, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%.

The *mpl* ligand isolated from aplastic porcine plasma has the following characteristics:

(1) The partially purified ligand elutes from a gel filtration column run in either PBS, PBS containing 0.1% SDS or PBS containing 4M $MgCl_2$ with Mr of 60,000-70,000;

(2) The ligand's activity is destroyed by pronase;

(3) The ligand is stable to low pH (2.5), SDS to 0.1%, and 2M urea;

(4) The ligand is a glycoprotein, based on its binding to a variety of lectin columns;

(5) The highly purified ligand elutes from non-reduced SDS-PAGE with a Mr of 25,000-35,000. Smaller amounts of activity also elute with Mr of ~18,000-22,000 and 60,000;

(6) The highly purified ligand resolves on reduced SDS-PAGE as a doublet with Mr of 28,000 and 31,000;

(7) The amino-terminal sequence of the 18,000-22,000, 28,000 and 31,000 bands is the same - SPAPPACDPRLLNKLLRDDHVLHGR (SEQ ID NO: 29); and

(8) The ligand binds and elutes from the following affinity columns

Blue-Sepharose,
CM Blue-Sepharose
MONO-Q,
MONO-S,
Lentil lectin-Sepharose,
WGA-Sepharose,
Con A-Sepharose,
Ether 650m Toyopearl,
Butyl 650 m Toyopearl,
Phenyl 650m Toyopearl, and
Phenyl-Sepharose.

More preferred *mpl* ligand polypeptides are those encoded by human genomic or cDNA having an amino acid sequence described in **Fig. 1** (SEQ ID NO: 1).

Other preferred naturally occurring biologically active *mpl* ligand polypeptides of this invention include prepro-*mpl* ligand, pro-*mpl* ligand, mature *mpl* ligand, *mpl* ligand fragments and glycosylation variants thereof.

Still other preferred polypeptides of this invention include *mpl* ligand sequence variants and chimeras. Ordinarily, preferred *mpl* ligand sequence variants and chimeras are biologically active *mpl* ligand variants that have an amino acid sequence having at least 70% amino acid sequence identity with the human *mpl* ligand or the *mpl* ligand isolated from aplastic porcine plasma, preferably at least 75%, more

preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95%. An exemplary preferred *mpl* ligand variant is a N-terminal domain hML variant (refered to as the "EPO-domain" because of its sequence homology to erythropoietin). The prefered hML EPO-domain comprises about the first 153 amino acid residues of mature hML and is refered to as hML_{153}. An optionally prefered hML sequence variant comprises one in which one or more of the basic or dibasic amino acid residue(s) in the C-terminal domain is substituted with a non-basic amino acid residue(s) (*e.g.*, hydrophobic, neutral, acidic, aromatic, Gly, Pro and the like). A prefered hML C-terminal domain sequence variant comprises one in which Arg residues 153 and 154 are replaced with Ala residues. This variant is refered to as hML_{332}(R153A, R154A). An alternative prefered hML variant comprises either hML_{332} or hML_{153} in which amino residues 111-114 (QLPP or LPPQ) are deleted or replaced with a diferent tetrapeptide sequence(*e.g.* AGAG or the like). The foregoing deletion mutants are refered to as $\Delta 4hML_{332}$ or $\Delta 4hML_{153}$.

A preferred chimera is a fusion between *mpl* ligand or fragment (defined below) thereof with a heterologous polypeptide or fragment thereof. For example, hML_{153} may be fused to an IgG fragment to improve serum half-life or to IL-3, G-CSF or EPO to produce a molecule with inhanced thrombopoietic or chimeric hematopoietic activity.

An alternative preferred human *mpl* ligand chimera is a "ML-EPO domain chimera" that consists of the N-terminus 153 to 157 hML residues substituted with one or more, but not all, of the human EPO residues approximately aligned as shown in **Fig. 10** (SEQ ID NO: 7). In this embodiment, the hML chimera would be about 153-166 residues in length in which individual or blocks of residues from the human EPO sequence are added or substituted into the hML sequence at positions corresponding to the alignment shown in **Fig. 10** (SEQ ID NO: 6). Exemplary block sequence inserts into the N-terminus portion of hML would include one or more of the N-glycosylation sites at positions (EPO) 24-27, 38-40, and 83-85; one or more of the four predicted amphipathic α-helical bundles at positions (EPO) 9-22, 59-76, 90-107, and 132-152; and other highly conserved regions including the N-terminus and C-terminus regions and residue positions (epo) 44-52 (see *e.g.*, Wen *et al.*, *Blood*, 82:1507-1516 [1993] and Boissel *et al.*, *J. Biol. Chem.*, **268(21)**:15983-15993 [1993]). It is contemplated this "ML-EPO domain chimera" will have mixed thrombopoietic-erythropoietic (TEPO) biological activity.

Other preferred polypeptides of this invention include *mpl* ligand fragments having a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of the *mpl* ligand isolated from aplastic

porcine plasma or the human *mpl* ligand described herein (see *e.g.* **Table 14, Example 24**). A preferred *mpl* ligand fragment is human ML[1-X] where X is 153, 164, 191, 205, 207, 217, 229, or 245 (see **Fig. 1** (SEQ ID NO: 1) for the sequence of residues 1-X). Other preferred *mpl* ligand fragments include those produced as a result of chemical or enzymatic hydrolysis or digestion of the purified ligand.

Another preferred aspect of the invention is a method for purifying *mpl* ligand molecules comprises contacting a *mpl* ligand source containing the *mpl* ligand molecules with an immobilized receptor polypeptide, specifically *mpl* or a *mpl* fusion polypeptide, under conditions whereby the *mpl* ligand molecules to be purified are selectively adsorbed onto the immobilized receptor polypeptide, washing the immobilized support to remove non-adsorbed material, and eluting the molecules to be purified from the immobilized receptor polypeptide with an elution buffer. The source containing the *mpl* ligand may be plasma where the immobilized receptor is preferably a *mpl*-IgG fusion.

Alternatively, the source containing the *mpl* ligand is recombinant cell culture where the concentration of *mpl* ligand in either the culture medium or in cell lysates is generally higher than in plasma or other natural sources. In this case the above described *mpl*-IgG immunoaffinity method, while still useful, is usually not necessary and more traditional protein purification methods known in the art may be applied. Briefly, the preferred purification method to provide substantially homogeneous *mpl* ligand comprises: removing particulate debris, either host cells or lysed fragments by, for example, centrifugation or ultrafiltration; optionally, protien may be concentrated with a commercially available protein concentration filter; followed by separating the ligand from other impurities by one or more steps selected from, immunoaffinity, ion-exchange (*e.g.*, DEAE or matrices containing carboxymethyl or sulfopropyl groups), Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toypearl, Butyl Toypearl, Phenyl Toypearl, protein A Sepharose, SDS-PAGE, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups) or Sephadex molecular seive or size exclusion chromatography, and ethanol or ammonium sulfate precipitation. A protease inhibitor such as methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis.

In another preferred embodiment, this invention provides an isolated antibody capable of binding to the *mpl* ligand. A preferred *mpl* ligand isolated antibody is monoclonal (Kohler and Milstein, *Nature*, 256:495-497 [1975]; Campbell, *Laboratory Techniques in Biochemistry and Molecular Biology*, Burdon *et al.*, Eds, Volume 13, Elsevier Science Publisrers, Amsterdam [1985]; and Huse *et al.*, *Science*, 246:1275-1281 [1989]). Preferred *mpl* ligand isolated antibody is one that binds

to *mpl* ligand with an affinity of at least about 10^6 l/mole. More preferably the antibody binds with an affinity of at least about 10^7 l/mole. Most preferably, the antibody is raised against the *mpl* ligand having one of the above described effector functions. The isolated antibody capable of binding to the *mpl* ligand may optionally be fused to a second polypeptide and the antibody or fusion thereof may be used to isolate and purify *mpl* ligand from a source as described above for immobilized *mpl* polypeptide. In a further preferred aspect of this embodiment, the invention provides a method for detecting the *mpl* ligand *in vitro* or *in vivo* comprising contacting the antibody with a sample, especially a serum sample, suspected of containing the ligand and detecting if binding has occurred.

In still further preferred embodiments, the invention provides an isolated nucleic acid molecule encoding the *mpl* ligand or fragments thereof, which nucleic acid molecule may be labeled or unlabeled with a detectable moiety, and a nucleic acid molecule having a sequence that is complementary to, or hybridizes under stringent or moderately stringent conditions with, a nucleic acid molecule having a sequence encoding a *mpl* ligand. A preferred *mpl* ligand nucleic acid is RNA or DNA that encodes a biologically active *mpl* ligand sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably 90%, and most preferably 95% sequence identity with the human *mpl* ligand. More preferred isolated nucleic acid molecules are DNA sequences encoding biologically active *mpl* ligand, selected from: (a) DNA based on the coding region of a mammalian *mpl* ligand gene (*e.g.*, DNA comprising the nucleotide sequence provided in **Fig. 1** (SEQ ID NO: 2), or fragments thereof); (b) DNA capable of hybridizing to a DNA of (a) under at least moderately stringent conditions; and (c) DNA that is degenerate to a DNA defined in (a) or (b) which results from degeneracy of the genetic code. It is contemplated that the novel *mpl* ligands described herein may be members of a family of ligands or cytokines having suitable sequence identity that their DNA may hybridize with the DNA of **Fig. 1** (SEQ ID NO: 2) (or the complement or fragments thereof) under low to moderate stringency conditions. Thus a further aspect of this invention includes DNA that hybridizes under low to moderate stringency conditions with DNA encoding the *mpl* ligand polypeptides.

In a further preferred embodiment of this invention, the nucleic acid molecule is cDNA encoding the *mpl* ligand and further comprises a replicable vector in which the cDNA is operably linked to control sequences recognized by a host transformed with the vector. This aspect further includes host cells transformed with the vector and a method of using the cDNA to effect production of *mpl* ligand, comprising expressing the cDNA encoding the *mpl* ligand in a culture of the transformed host cells and recovering the *mpl* ligand from the host cell culture. The *mpl* ligand prepared in this manner is

preferably substantially homogeneous human *mpl* ligand. A preferred host cell for producing *mpl* ligand is Chinese hamster ovary (CHO) cells.

The invention further includes a preferred method for treating a mammal having an immunological or hematopoietic disorder, especially thrombocytopenia comprising administering a therapeutically effective amount of a *mpl* ligand to the mammal. Optionally, the *mpl* ligand is administered in combination with a cytokine, especially a colony stimulating factor or interleukin. Preferred colony stimulating factors or interleukins include; kit-ligand, LIF, G-CSF, GM-CSF, M-CSF, EPO, IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 or IL-11.

III. Methods of Making

Platelet production has long been thought by some authors to be controlled by multiple lineage specific humoral factors. It has been postulated that two distinct cytokine activities, referred to as megakaryocyte colony-stimulating factor (meg-CSF) and thrombopoietin, regulate megakaryocytopoiesis and thrombopoiesis (Williams *et al., J. Cell Physiol.*, 110:101-104 [1982]; Williams *et al., Blood Cells*, 15:123-133 [1989]; and Gordon *et al., Blood*, 80:302-307 [1992]). According to this hypothesis, meg-CSF stimulates the proliferation of progenitor megakaryocytes while thrombopoietin primarily affects maturation of more differentiated cells and ultimately platelet release. Since the 1960's the induction and appearance of both meg-CSF and thrombopoietin activities in the plasma, serum and urine of animals and humans following thrombocytopenic episodes has been well documented (Odell *et al., Proc. Soc. Exp. Biol. Med.*, 108:428-431 [1961]; Nakeff *et al., Acta Haematol.*, 54:340-344 [1975]; Specter, *Proc Soc. Exp. Biol.*, 108:146-149 [1961]; Schreiner *et al., J.Clin.Invest.*, 49:1709-1713 [1970]; Ebbe, *Blood*, 44:605-608 [1974]; Hoffman *et al., N. Engl. J. Med.*, 305:533 [1981]; Straneva *et al., Exp. Hematol.*, 17:1122-1127 [1988]; Mazur *et al., Exp. Hematol.*, 13:1164 [1985]; Mazur *et al., J.Clin. Invest.*, 68:733-741 [1981]; Sheiner *et al., Blood*, 56:183-188 [1980]; Hill *et al., Exp. Hematol.*, 20:354-360 [1992]; and Hegyi *et al., Int. J. Cell Cloning*, 8:236-244 [1990]). These activities were reported to be lineage specific and distinct from known cytokines (Hill R.J. *et al., Blood* 80:346 (1992); Erickson-Miller C.L. *et al., Brit. J. Haematol.*, 84:197-203 (1993); Straneva J.E. *et al., Exp. Hematol.* 20:4750(1992); and Tsukada J. *et al., Blood* 81:866-867 [1993]). Heretofore, attempts to purify meg-CSF or thrombopoietin from thrombocytopenic plasma or urine have been unsuccessful.

Consistent with the above observations describing thrombocytopenic plasma, we have found that aplastic porcine plasma (APP) obtained from irradiated pigs stimulates human megakaryocytopoiesis *in vitro*. We have found that this stimulatory

activity is abrogated by the soluble extracellular domain of *c-mpl*, confirming APP as a potential source of the putative *mpl* ligand (ML). We have now successfully purified the *mpl* ligand from APP and amino acid sequence information was used to isolate murine, porcine and human ML cDNA. These ML's have sequence homology to erythropoietin and have both meg-CSF and thrombopoietin-like activities.

1. Purification and Identification of *mpl* Ligand from Plasma

As set forth above, aplastic plasma from a variety of species has been reported to contain activities that stimulate hematopoiesis *in vitro*, however no hematopoietic stimulatory factor has previously been reported isolated from plasma. One source of aplastic plasma is that obtained from irradiated pigs. This aplastic porcine plasma (APP) stimulates human hematopoiesis *in vitro*. To determine if APP contained the *mpl* ligand, its effect was assayed by measuring ^{3}H-thymidine incorporation into Ba/F3 cells transfected with human *mpl* P (Ba/F3-*mpl*) by the procedure shown in **Fig. 2**. APP stimulated ^{3}H-thymidine incorporation into Ba/F3-*mpl* cells but not Ba/F3 control cells (*i.e.*, not transfected with human *mpl* P). Additionally, no such activity was observed in normal porcine plasma. These results indicated that APP contained a factor or factors that transduced a proliferative signal through the *mpl* receptor and therefore might be the natural ligand for this receptor. This was futher supported by the finding that treatment of APP with soluble *mpl*-IgG blocked the stimulatory effects of APP on Ba/F3-*mpl* cells

The activity in APP appeared to be a protein since pronase, DTT, or heat destroy the activity in APP (**Fig. 3**). The activity was also non-dialyzable. The activity was, however, stable to low pH (pH 2.5 for 2 hrs) and was shown to bind and elute from several lectin-affinity columns, indicating that it was a glycoprotein. To further elucidate the structure and identity of this activity it was affinity purified from APP using a *mpl*-IgG chimera.

APP was treated according to the protocol set forth in **Examples 1** and **2**. Briefly, the *mpl* ligand was purified using hydrophobic interaction chromatography (HIC), immobilized dye chromatography, and *mpl*-affinity chromatography. The recovery of activity from each step is shown in **Fig. 4** and the fold purification is provided in **Table 1**. The overall recovery of activity through the *mpl*-affinity column was approximately 10%. The peak activity fraction (F6) from the *mpl*-affinity column has an estimated specific activity of 9.8 x10^{6} units/mg. The overall purification from 5 liters of APP was approximately 4 x10^{6} fold (0.8 units/mg to 3.3 x 10^{6} units/mg) with a 83 x 10^{6}-fold reduction in protein (250 gms to 3 μg). We estimated the specific activity of the ligand eluted from the *mpl*-affinity column to be ~3x10^{6} units/mg.

TABLE 1

Purification of *mpl* Ligand

Sample	Volume mls	Protein mg/ml	Units/ml	Units	Specific Acitivity Units/mg	Yield %	Fold Purification
APP	5000	50	40	200,000	0.8	-	1
Phenyl	4700	0.8	40	200,000	50	94	62
Blue-Sep.	640	0.93	400	256,000	430	128	538
mpl (μl) (Fxns 5-7)	12	$5x10^{-4}$	1666	20,000	3,300,000	10	4,100,000

Protein was determined by the Bradford assay. Protein concentration of *mpl*-eluted fractions 5-7 are estimates based on staining intensity of a silver stained SDS-gel. One unit is defined as that causing 50% maximal stimulation of Ba/F3-*mpl* cell proliferation.

Analysis of eluted fractions from the *mpl* affinity column by SDS-PAGE (4-20%, Novex gel) run under reducing conditions, revealed the presence of several proteins (Fig. 5). Proteins that silver stained with the strongest intensity resolved with apparent Mr of 66,000, 55,000 30,000, 28,000 and 18,000-22,000. To determine which of these proteins stimulated proliferation of Ba/F3-*mpl* cell cultures the proteins were eluted from the gel as described in **Example 2.**

The results of this experiment showed that most of the activity eluted from a gel slice that included proteins with Mr 28,000-32,000, with lesser activity eluting in the 18,000-22,000 region of the gel **(Fig. 6)**. The only proteins visible in these regions had Mr of 30,000, 28,000 and 18,000-22,000. To identify and obtain protein sequence for the proteins resolving in this region of the gel (*i.e.* bands at 30, 28 and 18-22 kDa), these three proteins were electroblotted to PVDF and sequenced as described in **Example 3.** Amino-terminus sequences obtained are provided in **Table 2**

TABLE 2

Mpl Ligand Amino-Terminus Sequences

30 kDa 1 5 10 15 20 25 (S) P A P P A(C)D P R L L N K L L R D D (H/S) V L H (G) R L	(SEQ ID NO: 30)
28 kDa 1 5 10 15 20 25 (S) P A P P A X D P R L L N K L L R D D (H) V L (H) G R	(SEQ ID NO: 31)
18-22 kDa 1 5 10 X P A P P A X D P R L X (N) (K)	(SEQ ID NO: 32)

Computer-assisted analysis revealed these amino acid sequences to be novel. Because all three sequences were the same, it was believed the 30 kDa, 28 kDa and 18-22 kDa proteins were related and might be different forms of the same novel protein. Futhermore, this protein(s) was a likely candidate as the natural *mpl* ligand because the activity resolved on SDS-PAGE in the same region (28,000-32,000) of a 4-20% gel. In addition, the partially purified ligand migrated with a Mr of 17,000-30,000 when subjected to gel filtration chromatography using a Superose 12 (Pharmacia) column. It is believed the different Mr forms of the ligand are a result of proteolysis or glycosylation differences or other post or pre-translational modifications

As described earlier, antisense human *mpl* RNA abrogated megakaryocytopoiesis in human bone marrow cultures enriched with CD 34^+ progenitor cells without affecting the differentiation of other hematopoietic cell lineages (Methia *et al., supra*). This result suggested that the *mpl* receptor might play a role in the differentiation and proliferation of megakaryocytes *in vitro*. To further elucidate the role of the *mpl* ligand in megakaryocytopoiesis, the effects of APP and *mpl* ligand depleted APP on *in vitro* human megakaryocytopoiesis was compared. The effect of APP on human megakaryocytopoiesis was determined using a modification of the liquid suspension megakaryocytopoiesis assay described in **Example 4**. In this assay, human peripheral stem cells (PSC) were treated with APP before and after *mpl*-IgG affinity chromatography. $GPII_bIII_a$ stimulation of megakaryocytopoiesis was quantitated with an ^{125}I-anti-II_bIII_a antibody (**Fig. 7**). Shown in **Fig. 7**, 10% APP caused approximately a 3-fold stimulation while APP depleted of *mpl* ligand had no effect. Significantly, the *mpl* ligand depleted APP did not induce proliferation of the Ba/F3-*mpl* cells.

In another experiment, soluble human *mpl*-IgG added at days 0, 2 and 4 to cultures containing 10% APP neutralized the stimulatory effects of APP on human megakaryocytopoiesis (**Fig. 8**). These results indicate that the *mpl* ligand plays a role in regulating human megakaryocytopoiesis and therefore may be useful for the treatment of thrombocytopenia.

2. Molecular Cloning of the *mpl* Ligand

Based on the amino-terminal amino acid sequence obtained from the 30 kDa, 28 kDa and 18-22 kDa proteins (see **Table 2** above), two degenerate oligonucleotide primer pools were designed and used to amplify porcine genomic DNA by PCR. It was reasoned that if the amino-terminal amino acid sequence was encoded by a single exon then the correct PCR product was expected to be 69 bp long. A DNA fragment of this size was found and subcloned into pGEMT The sequences of the oligonucleotide PCR primers and the three clones obtained are shown in **Example 5**. The amino acid sequence (PRLLNKLLR [SEQ ID NO 33]) of the peptide encoded between the PCR primers was identical to that obtained by amino-terminal protein sequencing of the porcine ligand (see residues 9-17 for the 28 and 30 kDa porcine protein sequences above).

A synthetic oligonucleotide based on the sequence of the PCR fragment was used to screen a human genomic DNA library. A 45-mer oligonucleotide, designated pR45, was designed and synthesized based on the sequence of the PCR fragment. This oligonucleotide had the following sequence

5' GCC-GTG-AAG-GAC-GTG-GTC-GTC-ACG-AAG-CAG-TTT-ATT-TAG-GAG-TCG 3'
(SEQ ID NO: 34)

This deoxyoligonucleotide was used to screen a human genomic DNA library in λgem12 under low stringency hybridization and wash conditions according to **Example 6**. Positive clones were picked, plaque purified and analyzed by restriction mapping and southern blotting. A 390 bp EcoRI-XbaI fragment that hybridized to the 45-mer was subcloned into pBluescript SK-. DNA sequencing of this clone confirmed that DNA encoding the human homolog of the porcine *mpl* ligand had been isolated. The human DNA sequence and deduced amino acid sequence are shown in **Fig. 9** (SEQ ID NOS: 3 & 4). The predicted positions of introns in the genomic sequence are also indicated by arrows, and define a putative exon ("exon 3").

Based on the human "exon 3" sequence (**Example 6**) oligonucleotides corresponding to the 3' and 5' ends of the exon sequence were synthesized. These 2 primers were used in PCR reactions employing as a template cDNA prepared from various human tissues. The expected size of the correct PCR product was 140 bp. After analysis of the PCR products on a 12% polyacrylamide gel, a DNA fragment of the

expected size was detected in cDNA libraries prepared from human adult kidney, 293 fetal kidney cells and cDNA prepared from human fetal liver.

A fetal liver cDNA library ($7x10^6$ clones) in lambda DR2 was next screened with the same 45-mer oligonucleotide used to screen the human genomic library and the fetal liver cDNA library under low stringency hybridization conditions. Positive clones were picked, plaque purified and the insert size was determined by PCR. One clone with a 1.8 kb insert was selected for further analysis. Using the procedures described in **Example 7** the nucleotide and deduced amino acid sequence of the human *mpl* ligand (hML) were obtained. These sequences are presented in **Fig. 1** (SEQ ID NOS: 1 & 2).

3. Structure of the Human *mpl* Ligand (hML)

The human *mpl* ligand (hML) cDNA sequence (**Fig. 1** [SEQ ID NO: 2]) comprises 1774 nucleotides followed by a poly(A) tail. It contains 215 nucleotides of 5' untranslated sequence and a 3' untranslated region of 498 nucleotides. The presumed initiation codon at nucleotide position (216-218) is within a consensus sequence favorable for eukaryotic translation initiation. The open reading frame is 1059 nucleotides long and encodes a 353 amino acid residue polypeptide, beginning at nucleotide position 220. The N-terminus of the predicted amino acid sequence is highly hydrophobic and probably corresponds to a signal peptide. Computer analysis of the predicted amino acid sequence (von Heijne *et al., Eur J. Biochem.*, **133**:17-21 [1983]) indicates a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage at that position would generate a mature polypeptide of 332 amino acid residues beginning with the amino-terminal sequence obtained from *mpl* ligand purified from porcine plasma. The predicted non-glycosylated molecular weight of the 332 amino acid residue ligand is about 38 kDa. There are 6 potential N-glycosylation sites and 4 cysteine residues.

Comparison of the *mpl* ligand sequence with the Genbank sequence database revealed 23% identity between the amino terminal 153 residues of mature human *mpl* ligand and human erythropoietin (**Fig. 10** [SEQ ID NOS: 6 & 7]). When conservative substitutions are taken into account, this region of hML shows 50% similarity to human erythropoietin (hEPO). Both hEPO and the hML contain four cysteines. Three of the 4 cysteines are conserved in hML, including the first and last cysteines. Site-directed mutagenesis experiments have shown that the first and last cysteines of erythropoietin form a disulfide bond that is required for function (Wang, F.F.*et al., Endocrinology* **116**:2286-2292 [1983]). By analogy, the first and last cysteines of hML may also form a critical disulfide bond. None of the glycosylation sites are

conserved in hML. All potential hML N-linked glycosylation sites are located in the carboxy-terminal half of the hML polypeptide.

Similar to hEPO, the hML mRNA does not contain the consensus polyadenylation sequence AAUAAA, nor the regulatory element AUUUA that is present in 3' untranslated regions of many cytokines and is thought to influence mRNA stability (Shaw*et al., Cell,* 46:659-667 [1986]). Northern blot analysis reveals low levels of a single 1.8 kb hML RNA transcript in both fetal and adult liver. After longer exposure, a weaker band of the same size could be detected in adult kidney. By comparison, human erythropoietin is expressed in fetal liver and, in response to hypoxia, the adult kidney and liver (Jacobs *et al.*, *Nature*, 313:804-809 [1985] and Bondurant *et al.*, *Molec. Cell. Biol.*, 6:2731-2733 [1986])

The importance of the C-terminal region of the hML remains to be elucidated. Based on the presence of the six potential sites for N-linked glycosylation and the ability of the ligand to bind lectin-affinity columns, this region of the hML is likely glycosylated. In some gel elution experiments, we observed activity resolving with a M_r around 60,000 which may represent the full length, glycosylated molecule. The C-terminal region may therefore act to stabilize and increase the half-life of circulating hML. In the case of erythropoietin, the non-glycosylated form has full *in vitro* biological activity, but has a significantly reduced plasma half-life relative to glycosylated erythropoietin (Takeuchi *et al.*, *J. Biol. Chem.*, 265:12127-12130 [1990]; Narhi *et al.*, *J. Biol. Chem.*, 266:23022-23026 [1991] and Spivack *et al.*, *Blood*, 7:90-99 [1989]). The C-terminal domain of hML contains two di-basic amino acid sequences [Arg-Arg motifs at positions 153-154 and 245-246] that could serve as potential processing sites. Cleavage at these sites may be responsible for generating the 30, 28 and 18-22 kDa forms of the ML isolated from APP. Significantly, the Arg_{153}-Arg_{154} sequence occurs immediately following the erythropoietin-like domain of the ML. These observations indicate that full length ML may represent a precursor protein that undergoes limited proteolysis to generate the mature ligand.

4. Isoforms and Variants of the Human *mpl* Ligand

Isoforms or alternatively spliced forms of human *mpl* ligand were detected by PCR in human adult liver. Briefly, primers were synthesized corresponding to each end as well as selected internal regions of the coding sequence of hML. These primers were used in RT-PCR to amplify human adult liver RNA as described in Example 10. In addition to the full length form, designated hML, three other forms, designated hML2, hML3 and hML4, were observed or deduced. The mature deduced amino acid sequences of all four isoforms is presented in Fig. 11 (SEQ ID NOS: 6, 8, 9 & 10).

hML3 has a 116 nucleotide deletion a position 700 which results in both an amino acid deletion and a frameshift. The cDNA now encodes a mature polypeptide that is 265 amino acid long and diverges from the hML sequence at amino acid residue 139. Finally, hML4 has both a 12 nucleotide deletion following nucleotide position 618 (also found in the mouse and the pig sequences [see below]) and the 116 bp deletion found in hML3. Altough no clones with only the 12 bp deletion (following nucleotide 619) have been isolated in the human (designated hML2), this form is likely to exist because such a isoform has been identified in both the mouse and pig (see below), and because it has been identified in conjunction with the116 nucleotide deletion in hML4.

Both a substitutional variant of hML in which the dibasic Arg_{153}-Arg_{154} sequence was replaced with two alanine residues and a "EPO-domain" truncated form of hML were constructed to determine whether the full length ML was necessary for biological activity. The Arg_{153}-Arg_{154} dibasic sequence substitutional variant, refered to as hML(R153A, R154A), was constructed using PCR as described in **Example 10**. The "EPO-domain" truncated form, hML_{153}, was also made using PCR by introducing a stop codon following Arg153.

5. Expression of Recombinant Human *mpl* Ligand (rhML) in Transiently Transfected Human Embryonic Kidney (293) Cells

To confirm that the cloned human cDNA encoded a ligand for *mpl*, the ligand was expressed in mammalian 293 cells under the control of the cytomegalovirus immediate early promoter using the expression vectors pRK5-hML or pRK5-hML_{153}. Supernatants from transiently transfected human embryonic kidney 293 cells were found to stimulate ^{3}H-thymidine incorporation in Ba/F3-*mpl* cells, but not in parental Ba/F3 cells (**Fig. 12A**). Media from the 293 cells transfected with the pRK vector alone did not contain this activity. Addition of *mpl*-IgG to the media abolished the stimulation (data not shown). These results show that the cloned cDNA encodes a functional human ML (hML).

To determine if the "EPO-domain" alone could bind and activate *mpl*, the truncated form of hML, $rhML_{153}$, was expressed in 293 cells. Supernatants from transfected cells were found to have activity similar to that present in supernatants from cells expressing the full length hML (**Fig. 12A**), indicating that the C-terminal domain of ML is not required for binding and activation of c-*mpl*.

6. *mpl* Ligand Stimulates Megakaryocytopoiesis and Thrombopoiesis

Both the full length rhML and the truncated $rhML_{153}$ forms of recombinant hML stimulated human megakaryocytopoiesis *in vitro* (**Fig. 12B**). This effect was observed in the absence of other exogenously added hematopoietic growth factors. With the exception of IL-3, the ML was the only hematopoietic growth factor tested that exhibited this activity. IL-11, IL-6, IL-1, erythropoietin, G-CSF, IL-9, LIF, kit ligand (KL), M-CSF, OSM and GM-CSF had no effect on megakaryocytopoiesis when tested separately in our assay (data not shown). This result demonstrates that the ML has megakaryocyte-stimulating activity and indicates a role for ML in regulating megakaryocytopoiesis.

Thrombopoietic activities present in plasma of thrombocytopenic animals have been shown to stimulate platelet production in a mouse rebound thrombocytosis assay (McDonald, *Proc. Soc. Exp. Biol Med.* 14:1006-1001 [1973] and McDonald *et al.*, *Scand J. Haematol.*, 16:326-334 [1976]). In this model mice are made acutely thrombocytopenic using specific antiplatelet serum, resulting in a predictable rebound thrombocytosis. Such immuno-thrombocythemic mice are more responsive to exogenous thrombopoietin-like activities than are normal mice (McDonald, *Proc. Soc. Exp. Biol. Med.*, 14:1006-1001 [1973]) just as exhypoxic mice are more sensitive to erythropoietin than normal are mice (McDonald, *et al.*, *J. Lab. Clin. Med.*, 77:134-143 [1971]). To determine whether the rML stimulates platelet production *in vivo*, mice in rebound thrombocytosis were injected with partially purified rhML. Platelet counts and incorporation of ^{35}S into platelets were then quantitated. Injection of mice with 64,000 or 32,000 units of rML significantly increased platelet production, as evidenced by a ~20% increase in platelet counts (p=0.0005 and 0.0001, respectively) and a ~40% increase in ^{35}S incorporation into platelets (p=0.003) in the treated mice versus control mice injected with excipient alone (**Fig. 12C**). This level of stimulation is comparable to that which we have observed with IL-6 in this model (data not shown). Treatment with 16,000 units of rML did not significantly stimulate platelet production. These results indicate that ML stimulates platelet production in a dose-dependent manner and therefore possesses thrombopoietin-like activity.

293 cells were also transfected with the other hML isoform constructs described above and the supernatants were assayed using the Ba/F3-*mpl* proliferation assay (see **Fig. 13**). hML2 and hML3 showed no detectable activity in this assay, however the activity of hML(R153A, R154A) was similar to hML and hML_{153} indicating that processing at the Arg_{153}-Arg_{154} di-basic site is neither required for nor detrimental to activity.

7. Megakaryocytopoiesis and the *mpl* Ligand

It has been proposed that megakaryocytopoiesis is regulated at multiple cellular levels (Williams *et al.*, *J.Cell Physiol.*, **110**:101-104 [1982] and Williams *et al.*, *Blood Cells*, **15**:123-133 [1989]). This is based largely on the observation that certain hematopoietic growth factors stimulate proliferation of megakaryocyte progenitors while others appear to primarily affect maturation. The results presented here suggest that the ML acts both as a proliferative and maturation factor. That ML stimulates proliferation of megakaryocyte progenitors is supported by several lines of evidence. First, APP stimulates both proliferation and maturation of human megakaryocytes *in vitro*, and this stimulation is completely inhibited by *mpl*-IgG (**Figs. 7 and 8**). Furthermore, the inhibition of megakaryocyte colony formation by c-*mpl* antisense oligonucleotides (Methia *et al.*, *Blood*, **82**:1395-1401 [1993]) and the finding that c-*mpl* can transduce a proliferative signal in cells into which it is transfected (Skoda *et al.*, *EMBO*, **12**:2645-2653 [1993] and Vigon *et al.*, *Oncogene*, **8**:2607-2615 [1993]) also indicate that ML stimulates proliferation. The apparent expression of c-*mpl* during all stages of megakaryocyte differentiation (Methia *et al.*, *Blood*, **82**:1395-1401 [1993]) and the ability of recombinant ML to rapidly stimulate platelet production *in vivo* indicate that ML also affects maturation. The availability of recombinant ML makes possible a careful evaluation of its role in regulating megakaryocytopoiesis and thrombopoiesis as well as its potential to influence other hematopoietic lineages

8. Isolation of the Human *mpl* Ligand (TPO) Gene

Human genomic DNA clones of the TPO gene were isolated by screening a human genomic library in λ-Gem12 with pR45, under low stringency conditions or under high stringency conditions with a fragment corresponding to the 3' half of human cDNA coding for the *mpl* ligand. Two overlapping lambda clones spanning 35 kb were isolated. Two overlapping fragments (BamH1 and EcoRI) containing the entire TPO gene were subcloned and sequenced (see **Figs. 14A, 14B and 14C**).

The structure of the human gene is composed of 6 exons within 7 kb of genomic DNA. The boundaries of all exon/intron junctions are consistent with the consensus motif established for mammalian genes (Shapiro, M. B., *et al.*, *Nucl. Acids Res.* **15**:7155 [1987]). Exon 1 and exon 2 contain 5' untranslated sequence and the initial four amino acids of the signal peptide. The remainder of the secretory signal and the first 26 amino acids of the mature protein are encoded within exon 3. The entire carboxyl domain and 3' untranslated as well as ~50 amino acids of the erythropoietin-

like domain are encoded within exon 6. The four amino acids involved in the deletion observed within hML-2 (hTPO-2) are encoded at the 5' end of exon 6.

Analysis of human genomic DNA by Southern blot indicated the gene for TPO is present in a single copy. The chromosomal location of the gene was determined by fluorescent *in situ* hybridization (FISH) which mapped to chromosome 3q27-28.

9. Expression and Purification of TPO from 293 Cells

Preperation and purification of ML or TPO from 293 cella is described in detail in **Example 19.** Briefly, cDNA corresponding to the TPO entire open reading frame was obtained by PCR using pRK5-h*mpl* I. The PCR product was purified and cloned between the restriction sites ClaI and XbaI of the plasmid pRK5tkneo (a pRK5 derived vector modified to express a neomycin resistance gene under the control of the thymidine kinase promote) to obtain the vector pRK5tkneo.ORF(a vector coding for the entire open reading frame).

A second vector coding for the EPO homologous domain was generated the same but using different PCR primers to obtain the final construct called pRK5-tkneoEPO-D.

These two constructs were transfected into Human Embryonic Kidney cells by the $CaPO_4$ method and neomycin resistant clones wer e selected and allowed to grow to confluency. Expression of ML_{153} or ML_{332} in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay.

Purification of $rhML_{332}$ was conducted as described in **Example 19.** Briefly, 293-$rhML_{332}$ conditioned media was applied to a Blue-Sepharose (Pharmacia) column that was subsequently washed with a buffer containing 2M urea. The column was eluted with a buffer containing 2M urea and 1M NaCl. The Blue-Sepharose elution pool was then directly applied to a WGA-Sepharose column, washed with 10 column volumes of buffer containing 2M urea and 1 M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-Sepharose eluate was applied to a C4-HPLC column (Synchrom, Inc.) and eluted with a discontinuous propanol gradient. By SDS-PAGE the purified 293-$rhML_{332}$ migrates as a broad band in the 68-80 kDa region of the gel(see **Fig. 15**).

Purification of $rhML_{153}$ was also conducted as described in **Example 19.** Briefly, 293-$rhML_{153}$ conditioned media was resolved on Blue-Sepharose as described for $rhML_{332}$. The Blue Sepharose eluate was applied directly to a *mpl*-affinity column as described above. $RhML_{153}$ eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC column run under the same conditions used for $rhML_{332}$. By SDS-PAGE the purified $rhML_{153}$ resolves into 2 major and 2 minor bands with Mr of ~18,000-22,000(see **Fig. 15**).

10. The Murine *mpl* Ligand

A DNA fragment corresponding to the coding region of the human *mpl* ligand was obtained by PCR, gel purified and labeled in the presence of ^{32}P-dATP and ^{32}P-dCTP. This probe was used to screen 10^6 clones of a mouse liver cDNA library in λGT10. A murine clone (**Fig. 16** [SEQ ID NOS: 12 & 13]) containing a 1443 base pair insert was isolated and sequenced. The presumed initiation codon at nucleotide position 138-141 was within a consensus sequence favorable for eukaryotic translation initiation (Kozak, M. *J.Cell Biol.*, **108**:229-241 [1989]). This sequence defines an open reading frame of 1056 nucleotides, which predicts a primary translation product of 352 amino acids. Flanking this open reading frame are 137 nucleotides of 5' and 247 nucleotides of 3' untranslated sequence. There is no poly(A) tail following the 3' untranslated region indicating that the clone is probably not complete. The N-terminus of the predicted amino acid sequence is highly hydrophobic and probably represents a signal peptide. Computer analysis (von Heijne, G. *Eur. J. Biochem.* **133**:17-21 [1983]) indicated a potential cleavage site for signal peptidase between residues 21 and 22. Cleavage at that position would generate a mature polypeptide of 331 amino acids (35 kDa) identified as mML_{331} (or mML2 for reasons described below). The sequence contains 4 cysteines, all conserved in the human sequence, and seven potential N-glycosylation sites, 5 of which are conserved in the human sequence. Again, as with hML, all seven potential N-glycosylation sites are located in the C-terminal half of the protein.

When compared with the human ML, considerable identity for both nucleotide and deduced amino acid sequences were observed in the "EPO-domains" of these ML's. However, when deduced amino acid sequences of human and mouse ML's were aligned, the mouse sequence appeared to have a tetrapeptide deletion between residues 111-114 corresponding to the 12 nucleotide deletion following nucleotide position 618 seen in both the human (see above) and pig (see below) cDNA's. Accordingly, additional clones were examined to detect possible murine ML isoforms. One clone encoded a 335 amino acid deduced sequence polypeptide containing the "missing" tetrapeptide LPLQ. This form is believed to be the full length murine ML and is refered to as mML or mML_{335}. The nucleotide and deduced amino acid sequence for mML are provided in Fig. 17 (SEQ ID NOS: 14 & 15). This cDNA clone consists of 1443 base pairs followed by a poly(A) tail. It possesses an open reading frame of 1068 bp flanked by 134 bases of 5' and 241 bases of 3' untranslated sequence. The presumed initiation codon lies at nucleotide position 138-140. The open reading frame encodes a predicted protein of 356 amino acids, the first 21 of which are highly hydrophobic and likely function as a secretion signal.

Finally, a third murine clone was isolated, sequenced and was found to contained the 116 nucleotide deletion corresponding to hML3. This murine isoform is therefore denominated mML3. Comparison of the deduced amino acid sequences of these two isoforms is shown in **Fig. 18** (SEQ ID NOS: 9 & 16).

The overall amino acid sequence identity between human and mouse ML (**Fig. 19** [SEQ ID NOS: 6 & 17]) is 72% but this homology is not evenly distributed. The region defined as the "EPO-domain" (amino acids 1-153 for the human sequence and 1-149 for the mouse) is better conserved (86% homology) than the carboxy-terminal region of the protein (62% homology). This may further indicate that only the "EPO-domain" is important for the biological activity of the protein. Interestingly, of the two di-basic amino acid motifs found in hML, only the di-basic motif immediately following the "EPO-domain" (residue position 153-154) in the human sequence is present in the murine sequence. This is consistent with the possibility that the full length ML may represent a precursor protein that undergoes limited proteolysis to generate the mature ligand. Alternatively, proteolysis between Arg_{153}-Arg_{154} may facilitate hML clearance

An expression vector containing the entire coding sequence of mML was transiently transfected into 293 cells as described in **Example 1**. Conditioned media from these cells stimulated ^{3}H-thymidine incorporation into Ba/F3 cells expressing either murine or human *mpl* but had no effect on the parental (*mpl*-less) cell line. This indicates that the cloned murine ML cDNA encodes a functional ligand that is able to activate both the murine and human ML receptor (*mpl*).

11. The Porcine *mpl* Ligand

Porcine ML (pML) cDNA was isolated by RACE PCR as described in **Example 13**. A PCR cDNA product of 1342 bp was found in kidney and subcloned. Several clones were sequenced and found to encode a pig *mpl* ligand of 332 amino acid resudues referred to as pML (or pML_{332}) having the nucleotide and deduced amino acid sequence shown in **Fig. 20** (SEQ ID NOS: 18 & 19).

Again, a second form, designated pML2, encoding a protein with a 4 amino acid residue deletion (228 amino acid residues) was identified (see **Fig. 21** [SEQ ID NO. 21]). Comparison of pML and pML2 amino acid sequences shows the latter form is identical except that the tetrapeptide QLPP corresponding to residues 111-114 inclusive have been deleted (see **Fig. 22** [SEQ ID NOS: 18 & 21]). The four amino acid deletions observed in both murine and porcine ML cDNA occur at precisely the same position within the predicted proteins.

Comparison of the predicted amino acid sequences of the mature ML from human, mouse, and pig (**Fig. 19** [SEQ ID NOS: 6, 17 & 18]) indicates that overall

sequence identity is 72 percent between mouse and human, 68 percent between mouse and pig and 73 percent between pig and human. The homology is substantially greater in the amino-terminal half of the ML (EPO homologous domain). This domain is 80 to 84 percent identical between any two species whereas the carboxy-terminal half (carbohydrate domain) is only 57 to 67 percent identical. A di-basic amino acid motif that could represent a protease cleavage site is present at the carboxyl end of the erythropoeitin homology domain. This motif is conserved between the three species at this position (**Fig. 19** [SEQ ID NOS: 6, 17 & 18]). A second di-basic site present at position 245 and 246 in the human sequence is not present in the mouse or pig sequences. The murine and the pig ML sequence contain 4 cysteines, all conserved in the human sequence. There are seven potential N-glycosylation sites within the mouse ligand and six within the porcine ML, 5 of which are conserved within the human sequence. Again, all the potential N-glycosylation sites are located in the C-terminal half of the protein.

12. Expression and Purification of TPO from Chinese Hamster Ovary (CHO) Cells

The expression vectors used to transfect CHO cells are designated pSVI5.ID.LL.MLORF (full length or TPO_{332}), and pSVI5.ID.LL.MLEPO-D (truncated or TPO_{153}). The pertinent features of these plasmids are presented in **Fig. 23 and 24**

The transfection procedures are described in **Example 20**. Briefly, cDNA corresponding to the entire open reading frame of TPO was obtained by PCR. The PCR product was purified and cloned between two restriction sites (ClaI and SalI) of the plasmid pSVI5.ID.LL to obtain the vector pSVI5.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using a different reverse primer(EPOD.Sal). The final construct for the vector coding for the EPO homologous domain of TPO is called pSVI5.ID.LL.MLEPO-D.

These two constructs were linearized with NotI and transfected into Chinese Hamster Ovary Cells (CHO-DP12 cells, EP 307,247 published 15 March 1989) by electroporation. 10^7 cells were electroporated in a BRL electroporation apparatus (350 Volts, 330 mF, low capacitance) in the presence of 10, 25 or 50 mg of DNA as described (Andreason, G.L. *J. Tissue Cult. Meth.* 15,56 [1993]). The day following transfection, cells were split in DHFR selective media (High glucose DMEM-F12 50:50 without glycine, 2mM glutamine, 2-5% dialyzed fetal calf serum). 10 to 15 days later individual colonies were transferred to 96 well plates and allowed to grow to confluency. Expression of ML_{153} or ML_{332} in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay (described in **Example I**).

The process for purifying and isolating TPO from harvested CHO cell culture fluid is described in **Example 20**. Briefly, harvested cell culture fluid (HCCF) is applied to a Blue Sepharose column (Phamacia) at a ratio of approximately 100L of HCCF per liter of resin. The column is then washed with 3 to 5 column volumes of buffer followed by 3 to 5 column volumes of a buffer containing 2.0M urea. TPO is then eluted with 3 to 5 column volumes of buffer containing both 2.0M urea and 1.0M NaCl.

The Blue Sepharose eluate pool containing TPO is then applied to a Wheat Germ Lectin Sepharose column (Pharmacia) equilibrated in the Blue Sepharose eluting buffer at a ratio of from 8 to 16 ml of Blue Sepharose eluate per ml of resin. The column is then washed with 2 to 3 column volumes of equilibration buffer. TPO is then eluted with 2 to 5 column volumes of a buffer containing 2.0M urea and 0.5M N-acetyl-D-glucosamine.

The Wheat Germ Lectin eluate containing TPO is then acidified and $C_{12}E_8$ is added to a final concentration of 0.04%. The resulting pool is applied to a C4 reversed phase column equilibrated in 0.1% TFA, 0.04% $C_{12}E_8$ at a load of approximately 0.2 to 0.5 mg protein per ml of resin

The protein is eluted in a two phase linear gradient of acetonitrile containing 0.1% TFA and 0.04% $C_{12}E_8$ and a pool is made on the basis of SDS-PAGE

The C4 Pool is then diluted and diafiltered versus approximately 6 volumes of buffer on an Amicon YM or like ultrafiltration membrane having a 10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be then directly processed or further concentrated by ultrafiltration. The diafiltrate/concentrate is usually adjusted to a final concentration of 0.01% Tween-80

All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the calculated column volume is then applied to a Sephacryl S-300 HR column (Pharmacia) equilibrated in a buffer containing 0.01% Tween-80 and chromatographed. The TPO containing fractions which are free of aggregate and proteolytic degradation products are then pooled on the basis of SDS-PAGE. The resulting pool is filtered and stored at 2-8°C.

13. Methods for Transforming and Inducing TPO Synthesis in a Microorganism and Isolating, Purifying and Refolding TPO Made Therein

Construction of *E. coli* TPO expression vectors is described in detail in **Example 21**. Briefly, plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 were all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for

high level translation initiation and rapid purification. The plasmids pMP210-1, -T8, -21, -22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy-terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in *E. coli* upon induction of the tryptophan promoter (Yansura, D. G. *et. al. Methods in Enzymology* (Goeddel, D. V., Ed.) 185:54-60, Academic Press, San Diego [1990]). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids

The above TPO expression plasmids were used to transform the *E. coli* using the $CaCl_2$ heat shock method (Mandel. M. *et al. J. Mol. Biol.*, 53:159-162, [1970]) and other procedures described in **Example 21**. Briefly. the transformed cells were grown first at 37°C until the optical density (600nm) of the culture reached approximately 2-3. The culture was then diluted and. after growth with aeration, acid was added. The culture was then allowed to continue growing with aeration for another 15 hours after which time the cells were harvested by centrifugation.

The Isolation, Purification and Refolding procedures given below for production of biologically active. refolded human TPO or fragments thereof is described in **Examples 22 and 23** can be applied for the recovery of any TPO variant including N and C terminal extended forms Other procedures suitable for refolding recombinant or synthetic TPO can be found in the following patents, Builder *et al.*, U.S Patent 4,511,502; Jones *et al.*, U.S. Patent 4,512,922; Olson U.S. Patent 4,518,526 and Builder *et al.*, U.S. Patent 4,620,948; for a general description of the recovery and refolding process for a variety of recombinant proteins expressed in an insoluble form in *E coli*.

A *Recovery of non-soluble TPO*

A microorganism such as *E. coli* expressing TPO encoded by any suitable plasmid is fermented under conditions in which TPO is deposited in insoluble "refractile bodies". Optionally, cells are first washed in a cell disruption buffer. Typically, about 100g of cells are resuspended in about 10 volumes of a cell disruption buffer (*e.g.* 10 mM Tris, 5 mM EDTA, pH 8) with, for example, a Polytron homogenizer and the cells centrifuged at 5000 x g for 30 minutes. Cells are then lysed using any conventional technique such as tonic shock, sonication, pressure cycling, chemical or enzymatic methods. For example, the washed cell pellet above may be resuspended in another 10 volumes of a cell disruption buffer with a homogenizer and the cell suspension is passed through an LH Cell Disrupter (LH Inceltech, Inc.) or through a Microfluidizer (Microfluidics International) according to the manufactures'

instructions. The particulate matter containing TPO is then separated from the liquid phase and optionally washed with any suitable liquid. For example, a suspension of cell lysate may be centrifuged at 5,000 X g for 30 minutes, resuspended and optionally centrifuged a second time to make a washed refractile body pellet. The washed pellet may be used immediately or optionally stored frozen (at *e.g.* -70 °C).

B. Solubilization and Purification of Monomeric TPO

Insoluble TPO in the refractile body pellet is then solubilized with a solublizing buffer. The solublizing buffer contains a chaotropic agent and is usually buffered at a basic pH and contains a reducing agent to improve the yield of monomeric TPO. Representative chaotropic agents include urea, guanidine·HCl, and sodium thiocyanate. A preferred chaotropic agent is guanidine·HCl. The concentration of chaotropic agent is usually 4-9M, preferably 6-8M. The pH of the solublizing buffer is maintained by any suitable buffer in a pH range of from about 7.5-9.5, preferably 8.0-9.0 and most preferably 8.0. Preferably the solubilizing buffer also contains a reducing agent to aid formation of the monomeric form of TPO Suitable reducing agents include organic compounds containing a free thiol (RSH). Representative reducing agents include dithiothreitol (DTT), dithioerythritol (DTE) mercaptoethanol, glutathione (GSH), cysteamine and cysteine. A preferred reducing agent is dithiothreitol (DTT). Optionally, the solubilizing buffer may contain a mild oxidizing agent (*e.g.* molecular oxygen) and a sulfite salt to form monomeric TPO via sulfitolysis. In this embodiment, the resulting TPO-S-sulfonate is later refolded in the presence of the redox buffer (*e.g.* GSH/GSSG) to form the properly folded TPO.

The TPO protein is usually further purified using, for example, centrifugation, gel filtration chromatography and reversed phase column chromatography.

By way of illustration, the following procedure has produced suitable yields of monomeric TPO The refractile body pellet is resuspended in about 5 volumes by weight of the solubilizing buffer (20 mM Tris, pH 8, with 6-8 M guanidine and 25 mM DTT) and stirred for 1-3 hr., or overnight, at 4 °C to effect solubilization of the TPO protein. High concentrations of urea (6-8M) are also useful but generally result in somewhat lower yields compared to guanidine. After solubilization, the solution is centrifuged at 30,000 x g for 30 min. to produce a clear supernatant containing denatured, monomeric TPO protein. The supernatant is then chromatographed on a Superdex 200 gel filtration column (Pharmacia, 2.6 x 60 cm) at a flow rate of 2 ml/min. and the protein eluted with 20 mM Na phosphate, pH 6.0, with 10 mM DTT. Fractions containing monomeric, denatured TPO protein eluting between 160 and 200 ml are pooled. The TPO protein is further purified on a semi-preparative C4 reversed phase column (2 x 20 cm VYDAC). The sample is applied at 5 ml/min. to a column equilibrated in 0.1% TFA(trifluoroacetic acid) with 30% acetonitrile. The protein is

eluted with a linear gradient of acetonitrile (30-60% in 60 min.). The purified reduced protein elutes at approximately 50% acetonitrile. This material is used for refolding to obtain biologically active TPO variant.

C Refolding TPO to Generate the Biologically Active Form

Following solubilization and further purification of TPO, the biologically active form is obtained by refolding the denatured monomeric TPO in a redox buffer. Because of the high potency of TPO (half maximal stimulation in the Ba/F3 assay is achieved at approximately 3 pg/ml), it is possible to obtain biologically active material utilizing many different buffer, detergent and redox conditions. However, under most conditions only a small amount of properly folded material (<10%) is obtained. For commercial manufacturing processes, it is desirable to have refolding yields at least 10%, more preferably 30-50% and most preferably >50%. Many different detergents including Triton X-100, dodecyl-beta-maltoside, CHAPS, CHAPSO, SDS, sarkosyl, Tween 20 and Tween 80, Zwittergent 3-14 and others were found suitable for producing at least some properly folded material. Of these however, the most preferred detergents were those of the CHAPS family (CHAPS and CHAPSO) which were found to work best in the refolding reaction and to limit protein aggregation and improper disulfide formation. Levels of CHAPS greater than about 1% were most preferred. Sodium chloride was required for the best yields, with the optimal levels between 0.1 M and 0.5M. The presence of EDTA (1-5 mM) in the redox buffer was preferred to limit the amount of metal-catalyzed oxidation (and aggregation) which was observed with some preparations. Glycerol concentrations of greater than 15% produced the optimal refolding conditions. For maximum yields, it was essential to have a redox pair in the redox buffer consisting of both an oxidized and reduced organic thiol (RSH). Suitable redox pairs include mercaptoethanol, glutathione (GSH), cysteamine, cysteine and their corresponding oxidized forms. Preferred redox pairs were glutathione(GSH):oxidized glutathione(GSSG) or cysteine:cystine. The most preferred redox pair was glutathione(GSH):oxidized glutathione(GSSG). Generally higher yields were observed when the mole ratio of oxidized member of the redox pair was equal to or in excess over the reduced member of the redox pair. pH values between 7.5 and about 9 were optimal for refolding of these TPO variants. Organic solvents (*e.g.* ethanol, acetonitrile, methanol) were tolerated at concentrations of 10-15% or lower. Higher levels of organic solvents increased the amount of improperly folded forms. Tris and phosphate buffers were generally useful. Incubation at 4 °C also produced higher levels of properly folded TPO.

Refolding yields of 40-60% (based on the amount of reduced and denatured TPO used in the refolding reaction) are typical for preparations of TPO that have been purified through the first C4 step. Active material can be obtained when less pure

preparations (*e.g.* directly after the Superdex 200 column or after the initial refractile body extraction) although the yields are less due to extensive precipitation and interference of non-TPO proteins during the TPO refolding process.

Since TPO contains 4 cysteine residues, it is possible to generate three different disulfide versions of this protein

version 1: disulfides between cysteine residues 1-4 and 2-3
version 2: disulfides between cysteine residues 1-2 and 3-4
version 3: disulfides between cysteine residues 1-3 and 2-4.

During the initial exploration in determining refolding conditions, several different peaks containing the TPO protein were separated by C4 reversed phase chromatography. Only one of these peaks had significant biological activity as determined using the Ba/F3 assay. Subsequently, the refolding conditions were optimized to yield preferentially that version. Under these conditions, the misfolded versions were less than 10-20% of the total monomeric TPO obtained from the solubilizing step.

The disulfide pattern for the biologically active TPO has been determined to be 1-4 and 2-3 by mass spectrometry and protein sequencing, where the cysteines are numbered sequentially from the amino-terminus. This cysteine cross-linking pattern is consistent with the known disulfide bonding pattern of the related molecule erythropoietin.

D *Biological Activity of Recombinant, Refolded TPO*

Refolded and purified TPO has activity in both *in vitro* and *in vivo* assays. For example, in the Ba/F3 assay, half-maximal stimulation of thymidine incorporation into the Ba/F3 cells for TPO (Met^{-1} 1-153) was achieved at 3.3 pg /ml (0.3 pM). In the *mpl* receptor-based ELISA, half-maximal activity occurred at 1.9 ng/ml (120 pM). In normal and myelosuppressed animals produced by near-lethal X-radiation, refolded TPO (Met^{-1} 1-153) was highly potent (activity was seen at doses as low as 30 ng/mouse) to stimulate the production of new platelets. Similar biological activity was observed for other forms of TPO refolded in accordance with the above described procedures (see **Figs. 25, 26 and 28**).

14. Methods for Measurement of Thrombopoietic Activity

Thrombopoietic activity may be measured in various assays including the Ba/F3 *mpl* ligand assay described in **Example 1**, an *in vivo* mouse platelet rebound synthesis assay, induction of platelet cell surface antigen assay as measured by an anti-platelet immunoassay (anti-$GPII_bIII_a$) for a human leukemia megakaryoblastic cell line (CMK) (see Sato *et al.*, *Brit. J. Heamatol.*, 72:184-190 [1989])(see also the liquid suspension megakaryocytopoiesis assay described in **Example 4**), and

induction of polyploidization in a megakaryoblastic cell line (DAMI) (see Ogura *et al., Blood*, **72(1)**:49-60 [1988]). Maturation of megakaryocytes from immature, largely non-DNA synthesizing cells, to morphologically identifiable megakaryocytes involves a process that includes appearance of cytoplasmic organelles, acquisition of membrane antigens ($GPII_bIII_a$), endoreplication and release of platelets as described in the background. A lineage specific promoter (*i.e.*, the *mpl* ligand) of megakaryocyte maturation would be expected to induce at least some of these changes in immature megakaryocytes leading to platelet release and alleviation of thrombocytopenia. Thus, assays were designed to measure the emergence of these parameters in immature megakaryocyte cell lines, *i.e.*, CMK and DAMI cells. The CMK assay (**Example 4**) measures the appearance of a specific platelet marker, $GPII_bIII_a$, and platelet shedding. The DAMI assay (**Example 15**) measures endoreplication since increases in ploidy are hallmarks of mature megakaryocytes Recognizable megakaryocytes have ploidy values of 2N, 4N, 8N, 16N, 32N, *etc*. Finally, the *in vivo* mouse platelet rebound assay (**Example 16**) is useful in demonstrating that administration of the test compound (here the *mpl* ligand) results in elevation of platelet numbers.

Two additional *in vitro* assays have been developed to measure TPO activity. The first is a **kinase receptor activation** (KIRA) ELISA in which CHO cells are transfected with a *mpl*-Rse chimera and tyrosine phosphorylation of Rse is measured by ELISA after exposure of the *mpl* portion of the chimera to *mpl* ligand (see **Example 17**) The second is a receptor based ELISA in which ELISA plate coated rabbit anti-human IgG captures human chimeric receptor *mpl*-IgG which binds the *mpl* ligand being assayed. A biotinylated rabbit polyclonal antibody to *mpl* ligand (TPO_{155}) is used to detect bound *mpl* ligand which is measured using streptavidin-peroxidase as described in **Example 18**.

15. *In Vivo* Biological Response of Normal and Sublethally Irradiated Mice Treated with TPO

Both normal and sublethally irradiated mice were treated with truncated and full length TPO isolated from Chinese hamster ovary (CHO) cells, *E. coli*, and human embryonic kidney (293) cells. Both forms of TPO produced in these three hosts stimulated platelet production in mice, however, full length TPO isolated from CHO apeared to produce the greatest *in vivo* response. These results indicate that proper glycosylation of the carboxy-terminal domain may be necessary for optimal *in vivo* activity.

(a) *E. coli*-rhTPO$(Met^{-1},153)$

The "Met" form of the EPO domain (Met in the -1 position plus the first 153 residues of human TPO) produced in *E. coli* (see **Example 23**) was injected daily into

normal female C57 B6 mice as described in the legends to **Fig. 25 A, B, and C.** These figures show that the non-glycosylated truncated form of TPO produced in *E. coli* and refolded as described above is capable of stimulating about a two-fold increase in platelet production in normal mice with out effecting the red or white blood cell population.

This same molecule injected daily into sublethally irradiated (^{137}Cs) female C57 B6 mice as described in the legends to **Fig. 26 A, B, and C** stimulated platelet recovery and diminished nadir but had no effect on erythrocytes or leukocytes.

(b) CHO-$rhTPO_{332}$

• The full length form of TPO produced in CHO and injected daily into normal female C57 B6 mice as described in the legends to **Fig. 27 A, B, and C** produced about a five-fold increase in platelet production in normal mice with out effecting the erythrocyte or leukocyte population.

(c) CHO-$rhTPO_{332}$. *E. coli*-$rhTPO_{(Met^{-1},153)}$; 293-$rhTPO_{332}$; and *E. coli*-$rhTPO_{155}$

Dose response curves were constructed for treatment of normal mice with rhTPO from various cell lines (CHO-$rhTPO_{332}$; *E. coli*-$rhTPO_{(Met^{-1},153)}$; 293-$rhTPO_{332}$; and *E. coli*-$rhTPO_{155}$) as described in the legend to **Fig. 28.** This figure shows that all tested forms of the molecule stimulate platelet production, however the full length form produced in CHO has the greatest *in vivo* activity

(d) CHO-$rhTPO_{153}$. CHO-rhTPO-"clipped" and CHO-$rhTPO_{332}$

Dose response curves were also constructed for treatment of normal mice with various forms of rhTPO produced in CHO (CHO-$rhTPO_{153}$. CHO-rhTPO-"clipped" and CHO-$rhTPO_{332}$) as described in the legend to **Fig. 29.** This figure shows that all tested CHO forms of the molecule stimulate platelet production,but that the full length 70 Kda form has the greatest *in vivo* activity.

16. General Recombinant Preparation of *mpl* Ligand and Variants

Preferably *mpl* ligand is prepared by standard recombinant procedures which involve production of the *mpl* ligand polypeptide by culturing cells transfected to express *mpl* ligand nucleic acid (typically by transforming the cells with an expression vector) and recovering the polypeptide from the cells. However, it is optionally envisioned that the *mpl* ligand may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the *mpl* ligand. For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element may be inserted in the genome of the intended host cell in

proximity and orientation sufficient to influence the transcription of DNA encoding the desired *mpl* ligand polypeptide. The control element does not encode the *mpl* ligand, rather the DNA is indigenous to the host cell genome. One next screens for cells making the receptor polypeptide of this invention, or for increased or decreased levels of expression, as desired.

Thus, the invention contemplates a method for producing *mpl* ligand comprising inserting into the genome of a cell containing the *mpl* ligand nucleic acid molecule a transcription modulatory element in sufficient proximity and orientation to the nucleic acid molecule to influence transcription thereof, with an optional further step comprising culturing the cell containing the transcription modulatory element and the nucleic acid molecule. The invention also contemplates a host cell containing the indigenous *mpl* ligand nucleic acid molecule operably linked to exogenous control sequences recognized by the host cell.

A. Isolation of DNA Encoding mpl ligand Polypeptide

The DNA encoding *mpl* ligand polypeptide may be obtained from any cDNA library prepared from tissue believed to possess the *mpl* ligand mRNA and to express it at a detectable level. The *mpl* ligand gene may also be obtained from a genomic DNA library or by *in vitro* oligonucleotide synthesis from the complete nucleotide or amino acid sequence

Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to the *mpl* ligand. For cDNA libraries suitable probes include oligonucleotides of about 20-80 bases in length that encode known or suspected portions of the *mpl* ligand cDNA from the same or different species: and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in Chapters 10-12 of Sambrook *et al., supra*.

An alternative means to isolate the gene encoding *mpl* ligand is to use PCR methodology as described in section 14 of Sambrook *et al., supra*. This method requires the use of oligonucleotide probes that will hybridize to DNA encoding the *mpl* ligand. Strategies for selection of oligonucleotides are described below.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably human or porcine kidney (adult or fetal) or liver cell lines. For example, human fetal

liver cell line cDNA libraries are screened with the oligonucleotide probes. Alternatively, human genomic libraries may be screened with the oligonucleotide probes.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually designed based on regions of the *mpl* ligand which have the least codon redundancy. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ATP (*e.g.*, $\gamma^{32}P$) and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Of particular interest is the *mpl* ligand nucleic acid that encodes a full-length *mpl* ligand polypeptide. In some preferred embodiments, the nucleic acid sequence includes the native *mpl* ligand signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence.

B. Amino Acid Sequence Variants of Native mpl ligand

Amino acid sequence variants of *mpl* ligand are prepared by introducing appropriate nucleotide changes into the *mpl* ligand DNA, or by *in vitro* synthesis of the desired *mpl* ligand polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence for the porcine *mpl* ligand. For example, carboxy terminus portions of the mature full length *mpl* ligand may be removed by proteolytic cleavage, either *in vivo* or *in vitro*, or by cloning and expressing a fragment or the DNA encoding full length *mpl* ligand to produce a biologically active variant. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired biological activity. The amino acid changes also may alter post-translational processes of the *mpl* ligand, such as changing the number or position of glycosylation sites. For the design of amino acid sequence variants of the *mpl* ligand, the location of the mutation site and the nature of the mutation will depend on the *mpl* ligand characteristic(s) to be modified. The sites for mutation can be modified individually or in series, *e.g.*, by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

A useful method for identification of certain residues or regions of the *mpl* ligand polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells, *Science*, 244:1081-1085 [1989]. Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by any, but preferably a neutral or negatively charged, amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed *mpl* ligand variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. For example, variants of the *mpl* ligand polypeptide include variants from the *mpl* ligand sequence, and may represent naturally occurring alleles (which will not require manipulation of the *mpl* ligand DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the *mpl* ligand characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Alternatively, amino acid sequence deletions for the *mpl* ligand may include a portion of or the entire carboxy-terminus glycoprotein domain. Amino acid sequence deletions may also include one or more of the first 6 amino-terminus residues of the mature protein. Optional amino acid sequence deletions comprise one or more residues in one or more of the loop regions that exist between the 'helical bundels". Contiguous deletions ordinarily are made in even numbers of residues, but single or odd numbers of deletions are within the scope hereof. Deletions may be introduced into regions of low homology among the *mpl* ligands that share the most sequence identity to modify the activity of the *mpl* ligand. Or deletions may be introduced into regions of low homology among human *mpl* ligand and other mammalian *mpl* ligand polypeptides that share the most sequence identity to the human *mpl* ligand. Deletions from a mammalian *mpl* ligand polypeptide in areas of substantial homology with other mammalian *mpl* ligands will be more likely to modify the biological activity of the *mpl* ligand more

significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of *mpl* ligands in the affected domain, *e.g.*, beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (*i.e.*, insertions within the mature *mpl* ligand sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. An exemplary preferred fusion is that of *mpl* ligand or fragment thereof and another cytokine or fragment thereof. Examples of terminal insertions include mature *mpl* ligand with an N-terminal methionyl residue, an artifact of the direct expression of mature *mpl* ligand in recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the mature *mpl* ligand molecule to facilitate the secretion of mature *mpl* ligand from recombinant hosts. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the *mpl* ligand molecule include the fusion to the N- or C-terminus of *mpl* ligand of immunogenic polypeptides (*i.e.*, not endogenous to the host to which the fusion is administered), *e.g.*, bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

A third group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the *mpl* ligand molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of *mpl* ligand and sites where the amino acids found in other analogues are substantially different in terms of side-chain bulk, charge, or hydrophobicity, but where there is also a high degree of sequence identity at the selected site among various *mpl* ligand species and/or within the various animal analogues of one *mpl* ligand member.

Other sites of interest are those in which particular residues of the *mpl* ligand obtained from various family members and/or animal species within one member are identical. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in **Table 3** under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more

substantial changes, denominated exemplary substitutions in **Table 3**, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 3

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Lys; Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln; Lys; Arg	Arg
Ile (I)	Leu, Val; Met; Ala; Phe; norleucine	Leu
Leu (L)	norleucine, ile, val, Met; Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Leu; Val; Ile; Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; norleucine	Leu

Substantial modifications in function or immunological identity of the *mpl* ligand are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, Met. Ala. Val, Leu. Ile;

(2) neutral hydrophilic: Cys, Ser. Thr:

(3) acidic: Asp, Glu;

(4) basic: Asn. Gln, His, Lys, Arg;

(5) residues that influence chain orientation: Gly. Pro; and

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in the molecule. These sites are identified by inspection of the encoded amino acid sequence, in the case of trypsin, *e.g.*, for an arginyl or lysinyl residue. When protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment any methionyl residues other than the starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accordance with **Table 3**) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites

Any cysteine residues not involved in maintaining the proper conformation of the *mpl* ligand also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. It has been found that the first and forth cysteines in the epo domain, numbered from the amino-terminus, are necessary for maintaining proper conformation but that the second and third are not. Accordingly, the second and third cysteines in the epo domain may be substituted.

Nucleic acid molecules encoding amino acid sequence variants of *mpl* ligand are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of *mpl* ligand polypeptide.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of *mpl* ligand DNA. This technique is well known in the art as described by Adelman *et al., DNA*, 2:183 [1983]. Briefly, *mpl*

ligand DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of *mpl* ligand. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the *mpl* ligand DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea *et al.*, *Proc. Natl. Acad. Sci. USA*, 75:5765 [1978].

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera *et al.*, *Meth. Enzymol.*, 153:3 [1987] Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press. NY 1989).

Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the *mpl* ligand, and the other strand (the original template) encodes the native, unaltered sequence of the *mpl* ligand. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template, except for the mutated bases is generated. In addition this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with *ExoIII* nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above

DNA encoding *mpl* ligand mutants with more than one amino acid to be substituted may be generated in one of several ways If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes Instead, one of two alternative methods may be employed

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid

substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of *mpl* ligand polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction mixture is denatured for five minutes at 100°C, placed briefly on ice, and then 1 μl *Thermus aquaticus (Taq)* DNA polymerase (5 units/μl, purchased from Perkin-Elmer Cetus) is added below the mineral oil layer.

The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows.

2 min. 55°C

30 sec. 72°C, then 19 cycles of the following:

30 sec. 94°C

30 sec. 55°C, and

30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50 vol), and ethanol precipitated. and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants. cassette mutagenesis, is based on the technique described by Wells *et al. Gene*. 34:315 [1985]. The starting material is the plasmid (or other vector) comprising the *mpl* ligand DNA to be mutated. The codon(s) in the *mpl* ligand DNA to be mutated are identified There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the *mpl* ligand DNA After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3 and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated *mpl* ligand DNA sequence.

C. Insertion of Nucleic Acid into a Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding native or variant *mpl* ligand polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the nucleic acid to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a

signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

The *mpl* ligand of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the *mpl* ligand DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native *mpl* ligand signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase, alpha factor, or acid phosphatase leaders, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (*i.e.*, the *mpl* ligand presequence that normally directs secretion of *mpl* ligand from its native mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other *mpl* ligand polypeptides or from the same *mpl* ligand from a different animal species, signal sequences from a *mpl* ligand, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another

organism for expression. For example. a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of *mpl* ligand DNA. However, the recovery of genomic DNA encoding *mpl* ligand is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the *mpl* ligand DNA.

(iii) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin neomycin, methotrexate, or tetracycline. (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media. *e.g.* the gene encoding D-alanine racemase for *Bacilli.*

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.*, **1**:327 [1982]) mycophenolic acid (Mulligan *et al.*, *Science*, **209**:1422 [1980]) or hygromycin Sugden *et al.*, *Mol. Cell. Biol.*, **5**:410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Examples of other suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the *mpl* ligand nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes *mpl* ligand polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the

chromosomes of successive generations of recombinant cells. Increased quantities of *mpl* ligand are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 [1980]. The transformed cells are then exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding *mpl* ligand This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060) Alternatively, host cells [particularly wild-type hosts that contain endogenous DHFR] transformed or co-transformed with DNA sequences encoding *mpl* ligand, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418 See U.S. Patent No 4,965,199

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 [1979]; Kingsman *et al.*, *Gene*, 7:141 [1979]; or Tschemper *et al.*, *Gene*, 10:157 [1980]). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85:12 [1977]). The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC No. 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the *mpl* ligand nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the *mpl* ligand nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in

temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to *mpl* ligand encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native *mpl* ligand promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the *mpl* ligand DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed *mpl* ligand as compared to the native *mpl* ligand promoter.

Promoters suitable for use with prokaryotic hosts include the ß-lactamase and lactose promoter systems (Chang *et al.*, *Nature*, **275**:615 [1978]; and Goeddel *et al.*, *Nature*, **281**:544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, **8**:4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA*, **80**:21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding *mpl* ligand (Siebenlist *et al.*, *Cell*, **20**:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding *mpl* ligand polypeptide.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, **255**:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.*, **7**:149 [1968]; and Holland, *Biochemistry*, **17**:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate

dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Mpl ligand transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the *mpl* ligand sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature*, **273**:113 [1978]; Mulligan and Berg, *Science*, **209**:1422-1427 [1980]; Pavlakis *et al.*, *Proc Natl. Acad. Sci. USA*, **78**:7398-7402 [1981]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *Hin*dIII E restriction fragment Greenaway *et al.*, *Gene*, **18**:355-360 [1982] A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S Patent No 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 See also Gray *et al.*, *Nature*, **295**:503-508 [1982] on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, **297**:598-601 [1982] on expression of human ß-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, **79**:5166-5170 [1982] on expression of the human interferon ß1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA*, **79**:6777-6781 [1982] on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the *mpl* ligand of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, **78**:993 [1981]) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, **3**:1108 [1983]) to the

transcription unit, within an intron (Banerji *et al.*, *Cell*, 33:729 [1983]), as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, a-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 [1982] on enhancing elements for activation of eukaryotic promoters The enhancer may be spliced into the vector at a position 5' or 3' to the *mpl* ligand encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding *mpl* ligand

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC No 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.*, 9:309 [1981] or by the method of Maxam *et al.*, *Methods in Enzymology*, 65:499 [1980].

(viii) Transient Expression Vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the *mpl* ligand polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp.

16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogues and variants of *mpl* ligand polypeptide that have *mpl* ligand polypeptide biological activity.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of *mpl* ligand in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature*, **293**:620-625 [1981]; Mantei *et al.*, *Nature*, **281**:40-46 [1979]; Levinson *et al.*; EP 117,060: and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of *mpl* ligand is pRK5 (EP 307,247 U. S. patent no. 5,258,287) or pSVI6B (PCT Publication No. WO 91/08291).

D. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryotic cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC No. 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC No. 31,537), and *E coli* W3110 (ATCC No. 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, *e.g.*, PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for *mpl* ligand encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, **290**:140 [1981]; EP 139,383 published 2 May 1985). *Kluyveromyces* hosts (U.S. Patent No. 4,943,529) such as, *e.g.*, *K. lactis* (Louvencourt *et al.*, *J. Bacteriol.*, 737 [1983]), *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, *yarrowia* [EP 402,226], *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, *J. Basic Microbiol.*, **28**:265-278 [1988]). *Candida*, *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, **76**:5259-5263 [1979]), and filamentous fungi such as, *e.g*, *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res.*

Commun., 112:284-289 [1983]; Tilburn *et al.*, *Gene*, 26:205-221 [1983]; Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:1470-1474 [1984]) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4:475-479 [1985]).

Suitable host cells for the expression of glycosylated *mpl* ligand are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, *e.g.*, Luckow *et al.*, *Bio/Technology*, 6:47-55 [1988]. Miller *et al.*, *Genetic Engineering*, Setlow *et al.*, eds.. Vol. 8 (Plenum Publishing 1986), pp. 277-279; and Maeda *et al.*, *Nature*, 315:592-594 [1985]. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the *mpl* ligand DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the *mpl* ligand is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the *mpl* ligand DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, *J. Mol. Appl. Gen.*, 1:561 [1982]. In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors [1973]). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 [1980]); monkey

kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, **383**:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, **23**:315 [1983] and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, **52**:456-457 [1978] is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, **130**:946 [1977] and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, **76**:3829 [1979]. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

E. Culturing the Host Cells

Prokaryotic cells used to produce the *mpl* ligand polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the *mpl* ligand of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10

(Sigma), Minimal Essential Medium ([MEM]. Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, **58**:44 [1979], Barnes and Sato. *Anal. Biochem.*. **102**:255 [1980], U.S. Patent No. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or copending U.S.S.N. 07/592,107 or 07/592,141, both filed on 3 October 1990, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

F. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, **77**:5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or

body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al., Am. J. Clin. Path.,* 75:734-738 [1980].

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native *mpl* ligand polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

G. Purification of mpl ligand Polypeptide

Mpl ligand preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When *mpl* ligand is expressed in a recombinant cell other than one of human origin, the *mpl* ligand is completely free of proteins or polypeptides of human origin. However, it is still usually necessary to purify *mpl* ligand from other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the *mpl* ligand *per se.* As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. Alternatively, a commercially available protein concentration filter (*e.g.*, Amicon or Millipore Pellicon ultrafiltration units) may be used. The *mpl* ligand may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the *mpl* ligand is membrane bound. *Mpl* ligand thereafter is purified from contaminant soluble proteins and polypeptides by salting out and exchange or chromatographic procedures employing various gel matrices. These matrices include; acrylamide, agarose, dextran, cellulose and others common to protein purification. Exemplary chromatography procedures suitable for protein purification include; immunoaffinity (*e.g.*, anti-h*mpl* ligand Mab), receptoraffinity (*e.g.*, *mpl*-IgG or protein A Sepharose), hydrophobic interaction chromatography (HIC) (*e.g.*, ether, butyl, or phenyl Toyopearl), lectin chromatography (*e.g.*, Con A-Sepharose, lentil-lectin-Sepharose), size exclusion (*e.g.*, Sephadex G-75), cation- and anion-exchange columns (*e.g.*, DEAE or carboxymethyl- and sulfopropyl-cellulose), and reverse-phase high performance liquid chromatography (RP-HPLC) (see *e.g.*, Urdal *et al., J. Chromatog.*, 296:171 [1984] where two sequential RP-HPLC steps are used to purify recombinant human

IL-2). Other purification steps optionally include; ethanol precipitation; ammonium sulfate precipitation; chromatofocusing; preparative SDS-PAGE, and the like.

Mpl ligand variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native *mpl* ligand, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a *mpl* ligand fusion with another protein or polypeptide, *e.g.*, a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-*mpl* ligand column can be employed to absorb the *mpl* ligand variant by binding it to at least one remaining immune epitope. Alternatively, the *mpl* ligand may be purified by affinity chromatography using a purified *mpl*-IgG coupled to a (preferably) immobilized resin such as Affi-Gel 10 (Bio-Rad, Richmond, CA) or the like, by means well known in the art. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native *mpl* ligand may require modification to account for changes in the character of *mpl* ligand or its variants upon expression in recombinant cell culture.

H. Covalent Modifications of mpl ligand Polypeptide

Covalent modifications of *mpl* ligand polypeptides are included within the scope of this invention. Both native *mpl* ligand and amino acid sequence variants of the *mpl* ligand may be covalently modified. One type of covalent modification included within the scope of this invention is a *mpl* ligand fragment. Variant *mpl* ligand fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis or by enzymatic or chemical cleavage of the full-length or variant *mpl* ligand polypeptide. Other types of covalent modifications of the *mpl* ligand or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of the *mpl* ligand or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful: the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal: chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione: and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking *mpl* ligand to a water-insoluble support matrix or surface for use in the method for purifying anti-*mpl* ligand antibodies, and *vice versa*. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable

intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the *mpl* ligand polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native *mpl* ligand, and/or adding one or more glycosylation sites that are not present in the native *mpl* ligand.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the *mpl* ligand polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native *mpl* ligand sequence (for O-linked glycosylation sites). For ease, the *mpl* ligand amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the *mpl* ligand polypeptide at preselected bases such that codons are generated that will translate into

the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of *mpl* Ligand."

Another means of increasing the number of carbohydrate moieties on the *mpl* ligand is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 [1981].

Removal of carbohydrate moieties present on the *mpl* ligand polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact Chemical deglycosylation is described by Hakimuddin, *et al.*, *Arch. Biochem. Biophys.*, 259:52 [1987] and by Edge *et al.*, *Anal. Biochem.*, 118:131 [1981] Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.*, 138:350 [1987].

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin *et al.*, *J. Biol. Chem.*, 257:3105 [1982]. Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of *mpl* ligand comprises linking the *mpl* ligand polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

It will be appreciated that some screening of the recovered *mpl* ligand variant will be needed to select the optimal variant for binding to a *mpl* and having the immunological and/or biological activity defined above. One can screen for stability in recombinant cell culture or in plasma (*e.g.*, against proteolytic cleavage). high affinity to a *mpl* member, oxidative stability, ability to be secreted in elevated yields, and the like. For example, a change in the immunological character of the *mpl* ligand polypeptide, such as affinity for a given antibody, is measured by a competitive-type

immunoassay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, or susceptibility to proteolytic degradation are assayed by methods well known in the art.

17. General Methods for Preparation of Antibodies to *mpl* Ligand

Antibody Preparation

(i) Polyclonal antibodies

Polyclonal antibodies to *mpl* ligand polypeptides or fragments are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the *mpl* ligand and an adjuvant. It may be useful to conjugate the *mpl* ligand or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glytaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the *mpl* ligand polypeptide or fragment, immunogenic conjugates or derivatives by combining 1 mg of 1 µg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for *mpl* ligand antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal boosted with the conjugate of the same *mpl* ligand, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the *mpl* ligand monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, *Nature*, 256:495 [1975], or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567 [Cabilly *et al.*]).

In the hybridoma method, a mouse or other appropriate host animal, such as hamster is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 [1984]; Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp.51-63, Marcel Dekker, Inc., New York, 1987).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against *mpl* ligand. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson & Pollard, *Anal. Biochem.*, 107:220 [1980].

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *supra*). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences. (Cabilly *et al., supra*; Morrison *et al., Proc. Nat. Acad. Sci.*, **81**:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for a *mpl* ligand and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

For diagnostic applications, the antibodies of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, *e.g.*, ^{125}I, ^{32}P, ^{14}C, or ^{3}H, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter, *et*

al., Nature, **144**:945 [1962]; David, *et al., Biochemistry*, **13**:1014 [1974]; Pain, *et al., J. Immunol. Meth.*, 40:219 [1981]; and Nygren, *J. Histochem. and Cytochem.*, **30**:407 [1982].

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (which may be a *mpl* ligand or an immunologically reactive portion thereof) to compete with the test sample analyte (*mpl* ligand) for binding with a limited amount of antibody. The amount of *mpl* ligand in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein (*mpl* ligand) to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David & Greene, U.S Patent No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme (*e.g.*, horseradish peroxidase).

(iii) Humanized and human antibodies

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, **321**:522-525 [1986]; Riechmann *et al., Nature*, **332**:323-327 [1988]; Verhoeyen *et al., Science*, **239**:1534-1536 [1988]), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al., supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which

some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the so called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, **151**:2296 [1993]; Chothia and Lesk, *J. Mol. Biol.*, **196**:901 [1987]). Another method uses a particular framework derived from the concensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA*, **89**:4285 [1992]; Presta *et al., J. Immnol.*, **151**:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding. For further details see U.S. application Serial No. 07/934,373 filed 21 August 1992, which is a continuation-in-part of application Serial No. 07/715,272 filed 14 June 1991.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*,

Proc. Natl. Acad. Sci. USA. **90**:2551-255 [1993]; Jakobovits *et al.*, *Nature*, **362**:255-258 [1993]; Bruggermann *et al.*. *Year in Immuno.*, **7**:33 [1993]. Human antibodies can also be produced in phage display libraries (Hoogenboom and Winter, J. Mol. Biol. 227, 381 [1991]; Marks *et al.*, J. Mol. Biol. 222, 581 [1991]).

(iv) Bispecific antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. Methods for making bispecific antibodies are known in the art.

Traditionally, the recombinant production of bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Millstein and Cuello, *Nature*. **305**:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in PCT publication No. WO 93/08829 (published 13 May 1993), and in Traunecker *et al.*, *EMBO*, **10**:3655-3659 [1991].

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2 and CH3 regions. It is preferred to have the first heavy chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an

immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in copending application Serial No. 07/931,811 filed 17 August 1992

For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology*, **121**:210 [1986].

(v) Heteroconjugate antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (PCT publication Nos. WO 91/00360 and WO 92/00373; EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

IV. Therapeutic Use of the Megakaryocytopoietic Protein *mpl* Ligand

The biologically active *mpl* ligand having hematopoietic effector function and referred to here as a megakaryocytopoietic or thrombocytopoietic protein (TPO) may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia (*e.g.*, aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the compounds of this invention as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia. Additionally, these megakaryocytopoietic proteins may be useful in treating myeloproliferative thrombocytotic diseases as well as thrombocytosis from inflammatory conditions and in iron deficiency.

Preferred uses of the megakaryocytopoietic or thrombocytopoietic protein (TPO) of this invention are in conjunction with myelotoxic chemotherapy, myeloablative chemotherapy, and thrombocytopenia due to bone marrow failure.

Still other disorders usefully treated with the megakaryocytopoietic proteins of this invention include defects or damage to platelets resulting from drugs, poisoning or activation on artificial surfaces. In these cases, the instant compounds may be employed to stimulate "shedding" of new "undamaged" platelets. For a more complete

list of useful applications, see the "Background" *supra*, especially section (a)-(f) and references cited therein.

The megakaryocytopoietic proteins of the instant invention may be employed alone or in combination with other cytokines, hematopoietins, interleukins, growth factors, or antibodies in the treatment of the above-identified disorders and conditions. Thus, the instant compounds may be employed in combination with other protein or peptide having thrombopoietic activity including; G-CSF, GM-CSF, LIF, M-CSF, IL-1, IL-3, erythropoietin (EPO), kit ligand, IL-6, and IL-11.

The megakaryocytopoietic proteins of the instant invention are prepared in a mixture with a pharmaceutically acceptable carrier. This therapeutic composition can be administered intravenously or through the nose or lung. The composition may also be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art. Briefly, dosage formulations of the compounds of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sobitol; counterions such as sodium and/or nonionic surfactants such as Tween, Pluronics or polyethyleneglycol.

About 0.5 to 500 mg of a compound or mixture of the megakaryocytopoietic protein as the free acid or base form or as a pharmaceutically acceptable salt, is compounded with a physiologically acceptable vehicle, carrier, excipient, binder, preservative, stabilizer, flavor, *etc.*, as called for by accepted pharmaceutical practice. The amount of active ingredient in these compositions is such that a suitable dosage in the range indicated is obtained.

Sterile compositions for injection can be formulated according to conventional pharmaceutical practice. For example, dissolution or suspension of the active compound in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may

be desired. Buffers, preservatives. antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.*, **15**:167-277 [1981] and Langer, *Chem. Tech.*, **12**:98-105 [1982] or poly(vinylalcohol)], polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, **22**:547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988)

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days. certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity Rational strategies can be devised for protein stabilization depending on the mechanism involved For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release megakaryocytopoietic protein compositions also include liposomally entrapped megakaryocytopoietic protein. Liposomes containing megakaryocytopoietic protein are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, **82**:3688-3692 [1985]; Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, **77**:4030-4034 [1980]; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal megakaryocytopoietic protein therapy.

The dosage will be determined by the attending physician taking into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other

medications and other relevant clinical factors. Typically, the daily regimen will range from 0.1-100 μg/kg body weight. Preferably the dosage will range from 0.1-50 μg/kg body weight. More preferably, the dosage will range from 1 to 5 μg/kg/day. Optionally, the dosage range will be the same as that of other cytokines, especially G-CSF, GM-CSF, and EPO. Therapeutically effective dosages may be determined by either *in vitro* or *in vivo* methods.

EXAMPLES

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and illustrative examples, make and utilize the present invention to the fullest extent. The following working examples therefore specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way of the remainder of the disclosure.

EXAMPLE 1

Partial Purification of the Porcine mpl Ligand

Platelet-poor plasma was collected from normal or aplastic anemic pigs. Pigs were rendered aplastic by irradiation with 900 cGy of total body irradiation using a 4mEV linear accelerator. The irradiated pigs were supported for 6-8 days with intramuscular injections of cefazolin Subsequently, their total blood volume was removed under general anesthesia heparinized, and centrifuged at 1800 x g for 30min. to make platelet-poor plasma The megakaryocyte stimulating activity was found to peak 6 days after irradiation

Aplastic porcine plasma obtained from irradiated pigs is made 4M with NaCl and stirred for 30 min. at room temperature. The resultant precipitate is removed by centrifugation at 3800 rpm in a Sorvall RC3B and the supernatant is loaded onto a Phenyl-Toyopearl column (220 ml) equilibrated in 10 mM $NaPO_4$ containing 4M NaCl. The column is washed with this buffer until A_{280} is <0.05 and eluted with dH_2O. The eluted protein peak is diluted with dH_2O to a conductivity of 15mS and loaded onto a Blue-Sepharose column equilibrated (240 ml) in PBS. Subsequently, the column is washed with 5 column volumes each of PBS and 10mM $NaPO_4$ (pH 7.4) containing 2M urea. Proteins are eluted from the column with 10mM $NaPO_4$ (pH 7.4) containing 2M urea and 1M NaCl. The eluted protein peak is made 0.01% octyl glucoside(n-octyl β-D-glucopyranoside) and 1 mM each with EDTA and Pefabloc (Boehinger Mannheim) and loaded directly onto tandemly linked CD4-IgG (Capon, D.J. *et al. Nature* 337:525-531 [1989]) and *mpl*-IgG Ultralink (Pierce) columns (see below). The CD4-IgG (2 ml) column is removed after the sample is loaded and the *mpl*-IgG (4 ml) column is washed with 10 column volumes each of PBS and PBS

containing 2 M NaCl and eluted with 0.1M glycine-HCl pH 2.25. Fractions are collected into 1/10th volume 1M Tris-HCl (pH 8.0).

Analysis of eluted fractions from the *mpl*-affinity column by SDS-PAGE (4-20%, Novex gel) run under reducing conditions, revealed the presence of several proteins (**Fig. 5**). Proteins that silver stain with the strongest intensity resolve with apparent Mr of 66,000, 55,000, 30,000, 28,000 and 14,000. To determine which of these proteins stimulate proliferation of Ba/F3-*mpl* cell cultures these proteins were eluted from the gel as described in **Example 2** below.

Ultralink Affinity Columns

10-20 mg of *mpl*-IgG or CD4-IgG in PBS are coupled to 0.5 grams of Ultralink resin (Pierce) as described by the manufacturer's instructions.

Construction and Expression of mpl-IgG

A chimeric molecule comprising the entire extracellular domain of human *mpl* (amino acids 1-491) and the Fc region of a human IgG1 molecule was expressed in 293 cells. A cDNA fragment encoding amino acids 1-491 of human *mpl* was obtained by PCR from a human megakaryocytic CMK cell cDNA library and sequenced. A ClaI site was inserted at the 5' end and a BstEII site at the 3' end. This fragment was cloned upstream of the IgGI Fc coding region in a Bluescript vector between the ClaI and the BstEII sites after partial digestion of the PCR product with BstEII because of two other BstEII sites present in the DNA encoding the extracellular domain of *mpl*. The BstEII site introduced at the 3' end of the *mpl* PCR product was designed to have the Fc region in frame with the *mpl* extracellular domain. The construct was subcloned into pRK5-tkneo vector between the ClaI and XbaI sites and transfected into 293 human embryonic kidney cells by the calcium phosphate method. The cells were selected in 0.4 mg/ml G418 and individual clones were isolated. *Mpl*-IgG expression from isolated clones was determined using a human Fc specific ELISA. The best expression clone had an expression level of 1-2 mg/ml of *mpl*-IgG.

Ba/F3 mpl P Expressing Cells

A cDNA corresponding to the entire coding region of human *mpl* P was cloned into pRK5-tkneo which was subsequently linearized with NotI and transfected into the IL-3 dependent cell line Ba/F3 by electroporation (1×10^7 cells, 9605F, 250Volts). Three days later selection was started in the presence of 2 mg/ml of G418. The cells were selected as pools or individual clones were obtained by limiting dilution in 96 well plates. Selected cells were maintained in RPMI containing 15% FBS, 1mg /ml G418, 20mM Glutamine, 10mM HEPES and 100 μg/ml of Pen-Strep. Expression of *mpl* P in selected clones was determined by FACS analysis using a anti-*mpl* P rabbit polyclonal antibody.

Ba/F3 mpl ligand Assay

The *mpl* ligand assay was conducted as shown is **Fig. 2**. To determine the presence of *mpl* ligand from various sources, the *mpl* P Ba/F3 cells were starved of IL-3 for 24 hours at a cell density of 5 x 10^5 cells/ml in a humidified incubator at 37°C in 5% CO_2 and air. Following IL-3 starvation the cells were plated out in 96 well culture dishes at a density of 50,000 cells in 200 µl of media with or without diluted samples and cultured for 24 hrs in a cell culture incubator. 20 µl of serum free RPMI media containing 1 µCi of ^{3}H-thymidine was added to each well for the last 6-8 hours. The cells were then harvested on 96 well GF/C filter plates and washed 5 times with water. The filters were counted in the presence of 40 µl of scintillation fluid (Microscint 20) in a Packard Top Count counter.

EXAMPLE 2

Highly Purified Porcine mpl Ligand

Gel Elution Protocol

Equal amounts of affinity purified *mpl* ligand (fraction 6 eluted from the *mpl*-IgG column) and 2X Laemmli sample buffer were mixed at room temperature without reducing agent and loaded onto a Novex 4-20% polyacrylamide gel as quickly as possible. The sample was not heated As a control, sample buffer without ligand was run in an adjacent lane. The gel was run at 4-6°C at 135 volts for approximately 2 1/4 hours The running buffer was initially at room temperature. The gel was then removed from the gel box and the plate on one side of the gel removed.

A replica of the gel was made on nitrocellulose as follows: A piece of nitrocellulose was wet with distilled water and carefully laid on top of the exposed gel face so air bubbles were excluded Fiducial marks were placed on the nitrocellulose and the gel plate so the replica could be accurately repositioned after staining. After approximately 2 minutes, the nitrocellulose was carefully removed, and the gel was wrapped in plastic wrap and placed in the refrigerator. The nitrocellulose was stained with Biorad's gold total protein stain by first agitating it in 3 x 10 ml 0.1% Tween 20 + 0.5 M NaCl + 0.1 M Tris-HCl pH 7.5 over approximately 45 minutes followed by 3 x 10 ml purified water over 5 minutes. The gold stain was then added and allowed to develop until the bands in the standards were visible. The replica was then rinsed with water, placed over the plastic wrap on the gel and carefully aligned with the fiducial marks. The positions of the Novex standards were marked on the gel plate and lines were drawn to indicate the cutting positions. The nitrocellulose and plastic wrap were then removed and the gel cut along the indicated lines with a sharp razor blade. The cuts were extended beyond the sample lanes so they could be used to determine the positions of the slices when the gel was stained. After the slices were removed, the

remaining gel was silver stained and the positions of the standards and the cut marks were measured. The molecular weights corresponding to the cut positions were determined from the Novex standards.

The 12 gel slices were placed into the cells in two Biorad model 422 electro-eluters. 12-14K molecular weight cutoff membrane caps were used in the cells. 50 mM ammonium bicarbonate + 0.05% SDS (approximately pH 7.8) was the elution buffer. One liter of buffer was chilled approximately 1 hour in a 4-6°C coldroom before use. Gel slices were eluted at 10 ma/cell (40 v initially) in a 4-6°C coldroom. Elution took approximately 4 hours. The cells were then carefully removed and the liquid above the frit removed with a pipet. The elution chamber was removed and any liquid above the membrane cap removed with a pipet. The liquid in the membrane cap was removed with a Pipetman and saved. Fifty µl aliquots of purified water were then placed in the cap, agitated and removed until all the SDS crystals dissolved. These washes were combined with the saved liquid above. Total elution sample volume was 300-500 µl per gel slice. Samples were placed in 10 mm Spectrapor 4 12-14K cutoff dialysis tubing which had been soaked several hours in purified water. They were dialyzed overnight at 4-6°C against 600 ml of phosphate buffered saline (PBS is approximately 4 mM in potassium) per 6 samples. The buffer was replaced the next morning and dialysis continued for 2.5 hours. Samples were then removed from the dialysis bags and placed in microfuge tubes. The tubes were placed on ice for 1 hour, microfuged at 14K rpm for 3 min. and the supernatants carefully removed from the precipitated SDS. The supernatants were then placed on ice for approximately 1 hour more and microfuged again for 4 min. The supernatants were diluted in phosphate buffered saline and submitted for the activity assay. Remaining samples were frozen at -70°C

EXAMPLE 3

Porcine mpl Ligand Microsequencing

Fraction 6 (2.6 ml) from the *mpl*-IgG affinity column was concentrated on a Microcon-10 (Amicon). In order to prevent the *mpl* ligand from absorbing to the Microcon, the membrane was rinsed with 1% SDS and 5 µl of 10 % SDS was added to fraction 6. Sample buffer (20 µl) of 2X was added to the fraction #6 after Microcon concentration (20 µl) and the total volume (40 µl) was loaded on a single lane of a 4-20 % gradient acrylamide gel (Novex). The gel was run following Novex protocol. The gel was then equilibrated for 5 min. prior to electroblotting in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, containing 10% methanol. Electroblotting onto Immobilon-PSQ membranes (Millipore) was carried out for 45 min. at 250 mA constant current in a BioRad Trans-Blot transfer

cell (32). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 in 40% methanol, 0.1% acetic acid for 1 min. and destained for 2-3 min. with 10% acetic acid in 50% methanol. The only proteins that were visible in the Mr 18,000-35,000 region of the blot had Mr of 30,000, 28,000 and 22,000.

Bands at 30, 28 and 22 kDa were subjected to protein sequencing. Automated protein sequencing was performed on a model 470A Applied Biosystem sequencer equipped with an on-line PTH analyzer. The sequencer was modified to inject 80-90% of the sample (Rodriguez, *J. Chromatogr.*, **350**:217-225 [1985]). Acetone (~12 µl/l) was added to solvent A to balance the UV absorbance. Electroblotted proteins were sequenced in the Blott cartridge. Peaks were integrated with Justice Innovation software using Nelson Analytical 970 interfaces. Sequence interpretation was performed on a VAX 5900 (Henzel *et al., J. Chromatogr.*, **404**:41-52 [1987]). N-terminal sequences (using one letter code with uncertain residues in parenthesis) and quantity of material obtained (in brackets) is presented in **Table 2'**.

TABLE 2'

Mpl Ligand Amino-Terminus Sequences

30 kDa [1.8 pmol] 1 5 10 15 20 25 (S) P A P P A(C)D P R L L N K L L R D D (H/S) V L H (G) R L	(SEQ ID NO: 30)
28 kDa [0.5 pmol] 1 5 10 15 20 25 (S) P A P P A X D P R L L N K L L R D D (H) V L (H) G R	(SEQ ID NO: 31)
18-22 kDa [0.5 pmol] 1 5 10 X P A P P A X D P R L X (N) (K)	(SEQ ID NO: 32)

EXAMPLE 4

Liquid Suspension Megakaryocytopoiesis Assay

Human peripheral stem cells (PSC) (obtained from consenting patients) were diluted 5 fold with IMDM media (Gibco) and centrifuged for 15 min. at room temp. at 800 x g. The cell pellets were resuspended in IMDM and layered onto 60% Percoll (density 1.077 gm/ml) (Pharmacia) and centrifuged at 800 x g for 30 min. The light density mononuclear cells were aspirated at the interface and washed 2x with IMDM and plated out at 1-2 x 10^6 cells/ml in IMDM containing 30% FBS (1 ml final volume) in 24 well tissue culture clusters (Costar). APP or *mpl* ligand depleted APP was added to 10% and cultures were grown for 12-14 days in a humidified incubator

at 37°C in 5% CO_2 and air. The cultures were also grown in the presence of 10% APP with 0.5 µg of *mpl*-IgG added at days 0, 2 and 4. APP was depleted of *mpl* ligand by passing APP through a *mpl*-IgG affinity column.

To quantitate megakaryocytopoiesis in these liquid suspension cultures, a modification of Solberg *et al.* was used and employs a radiolabeled murine IgG monoclonal antibody (HP1-1D) to GPIIbIIIa (provided by Dr. Nichols, Mayo Clinic). 100 µg of HP1-1D (see Grant, B. *et al., Blood* **69**:1334-1339 [1987]). was radiolabeled with 1mCi of $Na^{125}I$ using Enzymobeads (Biorad, Richmond, CA) as described by the manufacturer's instructions. Radiolabeled HP1-1D was stored at -70°C in PBS containing 0.01% octyl-glucoside. Typical specific activities were 1-2 x 10^6 cpm/µg (>95% precipated by 12.5% trichloroacetic acid).

Liquid suspension cultures were set up in triplicate for each experimental point. After 12-14 days in culture the 1ml cultures were transferred to 1.5ml eppendorf tubes and centrifuged at 800 x g for 10 min. at room temp. and the resultant cell pellets were resuspended in 100 µl of PBS containing 0.02% EDTA and 20% bovine calf serum. 10ng of ^{125}I-HP1-1D in 50 µl of assay buffer was added to the resuspended cultures and incubated for 60 min. at room temperature (RT) with occasional shaking. Subsequently cells were collected by centrifugation at 800 x g for 10 min. at RT and washed 2x with assay buffer. The pellets were counted for 1 min. in a gamma counter (Packard). Non-specific binding was determined by adding 1 µg of unlabeled HP1-1D for 60 min. before the addition of labeled HP1-1D. Specific binding was determined as the total ^{125}I-HP1-1D bound minus that bound in the presence of excess unlabeled HP1-1D.

EXAMPLE 5

Oligonucleotide PCR Primers

Based on the amino-terminal amino acid sequence obtained from the 30 kDa, 28 kDa and 18-22 kDa proteins, degenerate oligonucleotides were designed for use as polymerase chain reaction (PCR) primers (see **Table 4**). Two primer pools were synthesized, a positive sense 20 mer pool encoding amino acid residues 2-8 (*mpl* 1) and an anti-sense 21-mer pool complimentary to sequences encoding amino acids 18-24 (*mpl* 2).

TABLE 4

Degenerate Oligonucleotide Primer Pools

mpl 1:5' CCN GCN CCN CCN GCN TGY GA 3' (2,048-fold degenerate)	(SEQ ID NO: 35)
mpl 2:5' NCC RTG NAR NAC RTG RTC RTC 3' (2,048-fold degenerate)	(SEQ ID NO: 36)

Porcine genomic DNA, isolated from porcine peripheral blood lymphocytes, was used as a template for PCR. The 50 µl reaction contained: 0.8 µg of porcine genomic DNA in 10mM Tris-HCl (pH 8.3), 50mM KCl, 3mM $MgCl_2$, 100 µg/ml BSA, 400 µM dNTPs, 1 µM of each primer pool and 2.5 units of *Taq* polymerase. Initial template denaturation was at 94°C for 8 min. followed by 35 cycles of 45 seconds at 94°C, 1 min. at 55°C and 1 min. at 72°C. The final cycle was allowed to extend for 10 min. at 72°C. PCR products were separated by electrophoresis on a 12% polyacrylamide gel and visualized by staining with ethidium bromide. It was reasoned that if the amino-terminal amino acid sequence was encoded by a single exon then the correct PCR product was expected to be 69 bp. A DNA fragment of this size was eluted from the gel and subcloned into pGEMT (Promega) Sequences of three clones are shown below in **Table 5.**

TABLE 5

69 bp Porcine Genomic DNA Fragments

gemT3 5'CCAGCGCCGC CAGCCTGTGA CCCCCGACTC CTAAATAAAC TGCCTCGTGA 3'GGTCGCGGCG GTCGGACACT GGGGGCTGAG GATTTATTTG ACGGAGCACT TGACCACGTT CAGCACGGC **[69 bp]** (SEQ ID NO: 37) ACTGGTGCAA GTCGTGCCG (SEQ ID NO: 38)
gemT7 5'CCAGCACCTC CGGCATGTGA CCCCCGACTC CTAAATAAAC TGCTTCGTGA 3'GGTCGTGGAG GCCGTACACT GGGGGCTGAG GATTTATTTG ACGAAGCACT CGACCACGTC CATCACGGC **[69 bp]** (SEQ ID NO: 39) GCTGGTGCAG GTAGTGCCG (SEQ ID NO: 40)
gemT9 P R L L N K L L R (SEQ ID NO: 32) 5' CCAGCACCGCCGGCATGTGACCCCCGACTCCTAAATAAACTGCTTCGTGACG 3' GGTCGTGGCGGCCGTACACTGGGGGCTGAGGATTTATTTGACGAAGCACTGC ATCATGTCTATCACGGT 3' (SEQ ID NO: 41) TAGTACAGATAGTGCCA 5' (SEQ ID NO: 42)

The position of the PCR primers is indicated by the underlined bases. These results verify the N-terminal sequence obtained for amino acids 9-17 for the 30 kDa, 28 kDa and 18-22 kDa proteins and indicated that this sequence is encoded by a single exon of porcine DNA.

EXAMPLE 6

Human mpl Ligand Gene

Based on the results from **Example 5**, a 45-mer deoxyoligonucleotide, called pR45, was designed and synthesized to screen a genomic library. The 45-mer had the following sequence:

5' GCC-GTG-AAG-GAC-GTG-GTC-GTC-ACG-AAG-CAG-TTT-ATT-TAG-GAG-TCG 3' (SEQ ID NO: 28)

This oligonucleotide was ^{32}P-labeled with ($\gamma^{32}P$)-ATP and T4 kinase and used to screen a human genomic DNA library in λgem12 under low stringency hybridization and wash conditions (see **Example 7**). Positive clones were picked, plaque purified and analyzed by restriction mapping and southern blotting. Clone #4 was selected for additional analysis.

A 2.8 kb BamHI-XbaI fragment that hybridized to the 45-mer was subcloned into pBluescript SK-. Partial DNA sequencing of this clone was preformed using as primers oligonucleotides specific to the porcine *mpl* ligand DNA sequence. The sequence obtained confirmed that DNA encoding the human homolog of the porcine *mpl* ligand had been isolated. An EcoRI restriction site was detected in the sequence allowing us to isolate a 390 bp EcoRI-XbaI fragment from the 2.8 kb BamHI-XbaI and to subclone it in pBluescript SK-.

Both strands of this fragment were sequenced. The human DNA sequence and deduced amino acid sequence are shown in **Fig. 9** (SEQ ID NOS: 3 & 4). The predicted positions of introns in the genomic sequence are also indicated by arrows, and define a putative exon ("exon 3").

Examination of the predicted amino acid sequence confirms that a serine residue is the first amino acid of the mature *mpl* ligand, as determined from direct amino acid sequence analysis. Immediately upstream from this codon the predicted amino acid sequence is highly suggestive of a signal sequence involved in secretion of the mature *mpl* ligand. This signal sequence coding region is probably interrupted at nucleotide position 68 by an intron.

In the 3' direction the exon appears to terminate at nucleotide 196. This exon therefore encodes a sequence of 42 amino acids, 16 of which are likely to be part of a signal sequence and 26 of which are part of the mature human *mpl* ligand.

EXAMPLE 7

Full Length Human mpl Ligand cDNA

Based on the human "exon 3" sequence (**Example 6**) two non-degenerate oligonucleotides corresponding to the 3' and 5' ends of the "exon 3" sequence were synthesized (**Table 6**).

TABLE 6

Human cDNA Non-degenerate PCR Oligonucleotid Primers

Fwd primer: 5' GCT AGC TCT AGA AAT TGC TCC TCG TGG TCA TGC TTC T 3'	(SEQ ID NO: 43)
Rvs primer: 5 CAG TCT GCC GTG AAG GAC ATG G 3'	(SEQ ID NO: 44)

These two primers were used in PCR reactions employing as a template DNA from various human cDNA libraries or 1 ng of Quick Clone cDNA (Clonetech) from various tissues using the conditions described in the **Example 5**. The expected size of the correct PCR product was 140 bp After analysis of the PCR products on a 12% polyacrylamide gel, a DNA fragment of the expected size was detected in cDNA libraries prepared from adult kidney, 293 fetal kidney cells and cDNA prepared from human fetal liver (Clonetech cat. #7171-1)

A fetal liver cDNA library in λ DR2 (Clonetech cat. # HL1151x) was screened with the same 45 mer oligonucleotide used to screen the human genomic library. The oligonucleotide was labelled with (γ^{32}P)-ATP using T4 polynucleotide kinase. The library was screened under low stringency hybridization conditions. The filters were prehybridized for 2hr then hybridized with the probe overnight at 42°C in 20% formamide, 5xSSC, 10xDenhardt's, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg/ml of sonicated salmon sperm DNA for 16hr. Filters were then rinsed in 2xSSC and then washed once in 0.5xSSC, 0.1% SDS at 42°C. Filters were exposed overnight to Kodak X-Ray film. Positive clones were picked, plaque purified and the insert size was determined by PCR using oligonucleotides flanking the BamHI-XbaI cloning in λ DR2 (Clonetech cat. #6475-1). 5 μl of phage stock was used as a template source. Initial denaturation was for 7 min. at 94°C followed by 30 cycles of amplification (1 min. at 94°C, 1 min. at 52°C and 1.5 min. at 72°C). Final extention was for 15 min. at 72°C. Clone # FL2b had a 1.8kb insert and was selected for further analysis.

The plasmid pDR2 (Clonetech, λDR2 & pDR2 cloning and Expression System Library Protocol Handbook, p 42) contained within the λDR2 phage arms, was rescued as described per manufacturer's instructions (Clonetech, λDR2 & pDR2 cloning and Expression System Library Protocol Handbook, p 29-30). Restriction analysis of the

plasmid pDR2-FL2b with BamHI and XbaI indicated the presence of an internal BamHI restriction site in the insert approximately at position 650. Digestion of the plasmid with BamHI-XbaI cut the insert in two fragments, one of 0.65 kb and one of 1.15 kb. DNA sequence was determined with three different classes of template derived from the plasmid pDR2-FL2b. DNA sequencing of double-stranded plasmid DNA was carried out with the ABI373 (Applied Biosystems, Foster City, California) automated fluorescent DNA sequencer using standard protocols for dye-labeled dideoxy nucleoside triphosphate terminators (dye-terminators) and custom synthesized walking primers (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA*, **74**:5463-5467 [1977]; Smith *et al.*, *Nature*, **321**:674-679 [1986]). Direct sequencing of polymerase chain reaction amplified fragments from the plasmid was done with the ABI373 sequencer using custom primers and dye-terminator reactions Single stranded template was generated with the M13 Janus vector (DNASTAR, Inc., Madison, Wisconsin) (Burland *et al.*, *Nucl. Acids Res.*, **21**:3385-3390 [1993]) BamHI-XbaI (1.15 kb) and BamHI (0.65 kb) fragments were isolated from the plasmid pDR2-FL2b, the ends filled in with T4 DNA polymerase in the presence of deoxynucleotides, and then subcloned into the SmaI site of M13 Janus. Sequencing was carried out with standard protocols for dye-labeled M13 universal and reverse primers or walking primers and dye-terminators. Manual sequencing reactions were carried out on single strand M13 DNA using walking primers and standard dideoxy-terminator chemistry (Sanger *et al.*, *Proc. Natl. Acad Sci. USA*, **74**:5463-5467 [1977]). ^{33}P-labeled α-dATP and Sequenase (United States Biochemical Corp., Cleveland, Ohio) DNA sequence assembly was carried out with Sequencher V2.1b12 (Gene Codes Corporation, Ann Arbor, Michigan) The nucleotide and deduced sequences of hML are provided in **Fig. 1** (SEQ ID NO. 1).

EXAMPLE 8

Isolation of the Human mpl Ligand (TPO) Gene.

Human genomic DNA clones of the TPO gene were isolated by screening a human genomic library in λ-Gem12 with pR45, a previously described oligonucleotide probe under low stringency conditions (see **Example 7**) or under high stringency conditions with a fragment corresponding to the 3' half of human cDNA coding for the *mpl* ligand (from the BamH1 site to the 3' end). Two overlapping lambda clones spanning 35 kb were isolated. Two overlapping fragments (BamH1 and EcoRI) containing the entire TPO gene were subcloned and sequenced. The structure of the human gene is composed of 6 exons within 7 kb of genomic DNA (**Fig. 14 A, B and C**). The boundaries of all exon/intron junctions are consistent with the consensus motif established for mammalian genes (Shapiro M. B., *et al.*, *Nucl. Acids Res.* **15**:7155 [1987]). Exon 1 and exon 2 contain 5' untranslated sequence and the initial four

amino acids of the signal peptide. The remainder of the secretory signal and the first 26 amino acids of the mature protein are encoded within exon 3. The entire carboxyl domain and 3' untranslated as well as ~50 amino acids of the erythropoietin-like domain are encoded within exon 6. The four amino acids involved in the deletion observed within hML-2 (hTPO-2) are encoded at the 5' end of exon 6.

EXAMPLE 9

Transient Expression of Human mpl Ligand (hML)

In order to subclone the full length insert contained in pDR2-FL2b, the plasmid was digested with XbaI to completion, then partially digested with BamHI. A DNA fragment corresponding to the 1.8 kb insert was gel purified and subcloned in pRK5 (pRK5-h*mpl* I) (see U.S. Patent No. 5,258,287 for construction of pRK5) under the control of the cytomegalovirus immediate early promoter. DNA from the construct pRK5-h*mpl* I was prepared by the PEG method and transfected in Human embryonic kidney 293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4) and 10% fetal bovine serum. Cells were transfected by the calcium phosphate method as described (Gorman, C. [1985] in *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. II, pp. 143-190, IRL Press, Washington, D. C.). 36 h after transfection, the supernatant of the transfected cells was assayed for activity in the proliferation assay (see **Example I**). Supernatant of 293 cells transfected with pRK vector only gave no stimulation of the Ba/F3 or Ba/F3-*mpl* cells (**Fig. 12A**). Supernatant of cells transfected with pRK5-h*mpl* I had no effect on the Ba/F3 cells but dramatically stimulates the proliferation of Ba/F3-*mpl* cells (**Fig. 12A**), indicating that this cDNA encodes a functionally active human *mpl* ligand.

EXAMPLE 10

Human Mpl Ligand Isoforms
hML2, hML3, and hML4

In order to identify alternatively spliced forms of hML, primers were synthesized corresponding to each end of the coding sequence of hML. These primers were employed in RT-PCR to amplify human adult liver RNA. Additionally, internal primers flanking selected regions of interest (see below) were constructed and similarly employed. Direct sequencing of the ends of the PCR product revealed a single sequence corresponding exactly to the sequence of the cDNA isolated from the human fetal liver library (see **Fig. 1** [SEQ ID NO: 1]). However, a region near the C-terminus of the EPO-domain (in the middle of the PCR product) exhibited a complex sequence pattern sugesting the existence of possible splice variants in that region. To

isolate these splice variants, the primers provided in **Table 7** flanking the region of interest were used in a PCR as templates for human adult liver cDNA.

TABLE 7

Human ML Isoform PCR Primers

phmplicdna.3e1:	5'TGTGGACTTTAGCTTGGGAGAATG3'	(SEQ ID NO: 45)
pbx4.f2:	5'GGTCCAGGGACCTGGAGGTTTG3'	(SEQ ID NO: 46)

The PCR products were subcloned blunt into M13. Sequencing of individual subclones revealed the existence of at least 3 ML isoforms. One of them, hML (also refered to as hML_{332}), is the longest form and corresponds exactly to the sequence isolated from the fetal liver library. Sequences of the four human *mpl* ligand isoforms listed from longest (hML) to shortest (hML-4) are provided in (**Fig. 11** [SEQ ID NOS: 6, 8, 9 & 10]).

EXAMPLE 11

Construction and Transient Expression of Human Mpl Ligand Isoforms and Substitutional Variants

hML2, hML3, and hML(R153A, R154A)

Isoforms hML2 and hML3 and substitutional variant hML(R153A, R154A) were reconstituted from hML using the recombinant PCR technique described by Russell Higuchi, in PCR Protocols, *A guide to Methods and Applications*, Acad. Press, M.A.Innis, D.H. Gelfand, J.J. Sninsky & T.J White Editors.

In all contructs, the "outside" primers used are shown in **Table 8** and the "overlapping" primers are shown in **Table 9**.

TABLE 8

Outside Primers

Cla.FL.F2: 5'ATC GAT ATC GAT AGC CAG ACA CCC CGG CCA G3'	(SEQ ID NO: 47)
HMPLL-R: 5'GCT AGC TCT AGA CAG GGA AGG GAG CTG TAC ATG AGA3'	(SEQ ID NO: 48)

TABLE 9

Overlapping Primers

hML-2:		
MLΔ4.F:	5'CTC CTT GGA ACC CAG GGC AGG ACC 3'	(SEQ ID NO: 49)
MLΔ4.R	5'GGT CCT GCC CTG GGT TCC AAG GAG 3'	(SEQ ID NO: 50)
hML-3:		
hMLΔ116+:	5'CTG CTC CGA GGA AAG GAC TTC TGG ATT 3'	(SEQ ID NO: 51)
hMLΔ116-:	5'AAT CCA GAA GTC CTT TCC TCG GAG CAG 3'	(SEQ ID NO: 52)
hML(R153A, R154A):		
RR-KO-F:	5'CCC TCT GCG TCG CGG CGG CCC CAC CCA C 3'	(SEQ ID NO: 53)
RR-KO-R:	5'GTG GGT GGG GCC GCC GCG ACG CAG AGG G 3'	(SEQ ID NO: 54)

All PCR amplifications were performed with cloned Pfu DNA polymerase (Stratagene) using the following conditions: Initial template denaturation was at 94°C for 7 min. followed by 30 cycles of 1 min at 94°C, 1 min. at 55°C and 1.5 min. at 72°C. The final cycle was allowed to extend for 10 min. at 72°C. The final PCR product was digested with ClaI-XbaI, gel purified and cloned in pRK5tkneo. 293 cells were transfected with the various constructs as described above and the supernatant was assayed using the Ba/F3-*mpl* proliferation assay hML-2 and hML-3 showed no detectable activity in this assay, however the activity of hML(R153A, R154A) was similar to hML indicating that processing at this di-basic site is not required for activity (see **Fig. 13**).

EXAMPLE 12

Murine mpl Ligand cDNA
mML, mML-2 and mML-3

Isolation of mML cDNA.

A DNA fragment corresponding to the entire coding region of the human *mpl* ligand was obtained by PCR, gel purified and labeled by random priming in the presence of ^{32}P-dATP and ^{32}P-dCTP. This probe was used to screen 10^6 clones of a mouse liver cDNA library in λGT10 (Clontech cat# ML3001a). Duplicate filters were hybridized in 35% formamide, 5xSSC, 10xDenhardt's, 0.1% SDS, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 100 µg/ml of sonicated salmon sperm DNA overnight in the presence of the probe. Filters were rinsed in 2xSSC and then washed once in 0.5xSSC, 0.1% SDS at 42°C. Hybridizing phage were plaque-purified and the cDNA inserts were subcloned into the Eco R1 site of Bluescript SK- plasmid. Clone "LD" with a 1.5 kb insert was chosen for further analysis and both strands were sequenced as descibed above for the human ML cDNA. The nucleotide and

deduced amino acid sequences from clone LD are provided in **Fig. 14** (SEQ ID NOS: 1 & 11). The deduced mature ML sequence from this clone was 331 amino acid residues long and identified as mML_{331} (or mML-2 for reasons described below). Considerable identity for both nucleotide and deduced amino acid sequences were observed in the EPO-like domains of these ML's. However, when deduced amino acid sequences of human and mouse ML's were aligned, the mouse sequence appeared to have a tetrapeptide deletion between human residues 111-114 corresponding to the 12 nucleotide deletion following nucleotide position 618 seen in both the human (see above) and pig (see below) cDNA's. Accordingly, additional clones were examined to detect possible murine ML isoforms. One clone, "L7", had a 1.4 kb insert with a 335 amino acid deduced sequence containing the "missing" tetrapeptide LPLQ. This form is believed to be the full length murine ML and is refered to as mML or mML_{335}. The nucleotide and deduced amino acid sequence for mML are provided in **Fig. 16** (SEQ ID NOS: 12 & 13). Finally, clone "L2" was isolated and sequenced. This clone has the 116 nucleotide deletion corresponding to hML3 and is therefore denominated mML-3. Comparison of the deduced amino acid sequences of these two isoforms is shown in **Fig. 16**.

Expression of recombinant mML Expression vectors for murine ML were prepared essentially as described in **Example 8**. Clones encoding mML and mML-2 were subcloned into pRK5tkneo, a mammalian expression vector that provides expression under the control of the CMV promoter and an SV40 polyadenylation signal. The resulting expression vectors, mMLpRKtkneo and mML2pRKtkneo were transiently transfected into 293 cells using the calcium phosphate method. Following transient transfection, media was conditioned for five days. The cells were maintained in high glucose DMEM media supplemented with 10% fetal calf serum.

Expression of murine-mpl (mmpl) in Ba/F3 cells. Stable cell lines expressing c-*mpl* were obtained by transfection of m*mpl* pRKtkneo, essentially as described for human *mpl* in **Example 1**. Briefly, an expression vector (20 µg; linearized) containing the entire coding sequence of murine *mpl* (Skoda, R. C., *et al.*, *EMBO J.* **12**:2645-2653 [1993]) was transfected into Ba/F3 cells by electroporation (5 X 10^6 cells, 250 volts, 960 µF) followed by selection for neomycine resistance with 2 mg/ml G418. Expression of *mpl* was assessed by flow cytometry analysis using rabbit anti-murine *mpl*-IgG antisera. Ba/F3 cells were maintained in RPMI 1640 media from WEHI -3B cells as a source of IL-3. Supernatants from 293 cells transiently transfected with both mML and mML-2 were assayed in BaF3 cells transfected with both m*mpl* and h*mpl* as described in **Example 1**.

EXAMPLE 13

Porcine mpl Ligand cDNA

pML and pML-2

Porcine ML (pML) cDNA was isolated by RACE PCR. Briefly, an oligo dT primer and 2 specific primers were designed based on the sequence of the exon of the porcine ML gene encoding the amino terminus of the ML purified from the aplastic pig serum. cDNA prepared from various aplastic pig tissues was obtained and amplified. A PCR cDNA product of 1342 bp was found in kidney and subcloned. Several clones were sequenced and found to encode the mature pig *mpl* ligand (not including a complete secretion signal). The cDNA was found to encode a 332 amino acid mature protein (pML_{332}) having the sequence shown in **Fig. 18** (SEQ ID NOS: 9 & 16).

Method:

Isolation of pML gene and cDNA. Genomic clones of the porcine ML gene were isolated by screening a pig genomic library in EMBL3 (Clontech Inc.) with pR45. The library was screened essentially as described in **Example 7.** Several clones were isolated and the exon encoding amino acid sequence identical to that obtained from the purified ML was sequenced. Porcine ML cDNA were obtained using a modification of the RACE PCR protocol. Two specific ML primers were designed based on the sequence of the pig ML gene. Polyadenylated mRNA was isolated from the kidney of aplastic pigs essentially as previously described. cDNA was prepared by reverse transcription with the BamdT primer

(BamdT: 5' GACTCGAGGATCCATCGATTTTTTTTTTTTTTTTTT 3')

(SEQ ID NO: 55)

directed against the polyadenosine tail of the mRNA. An initial round of PCR amplification (28 cycles of 95°C for 60 seconds, 58°C for 60 seconds, and 72°C for ninety seconds) was conducted using the ML specific h-forward-1 primer

(h-forward-1: 5' GCTAGCTCTAGAAATTGCTCCTCGTGGTCATGCTTCT 3')

(SEQ ID NO: 43)

and the BAMAD primer

(BAMAD: 5' GACTCGAGGATCCATCG 3')

(SEQ ID NO: 56)

in a 100 ml reaction (50 mM KCl, 1.5 mM MgCl, 10 mM Tris pH 8.0, 0.2 mM dNTPs,with 0.05 U/ml Amplitaq polymerase [Perkin Elmer Inc.]) The PCR product was then digested with Cla1, extracted with phenol-chloroform (1:1), ethanol precipitated, and ligated to 0.1 mg of Bluescript SK- vector (Stratagene inc.) that had been cut with Cla1 and Kpn 1. After incubation for two hours at room temperature, one fourth of the ligation mixture was added directly to a second round of PCR (22 cycles as described above) using a second ML specific forward-1 primer

(forward-1: 5' GCTAGCTCTAGAAGCCCGGCTCCTCCTGCCTG 3')

(SEQ ID NO: 57)

and T3-21 (an oligonucleotide that binds to a sequence adjacent to the multiple cloning region within the Bluescript SK- vector)

(5' CGAAATTAACCCTCACTAAAG 3')

(SEQ ID NO: 58).

The resulting PCR product was digested with Xba1 and Cla1 and subcloned into Bluescript SK-. Several clones from indepedent PCR reactions were sequenced.

Again, a second form, designated pML-2, encoding a protein with a 4 amino acid residue deletion (328 amino acid residues) was identified (see **Fig. 21** [SEQ ID NO: 21]). Comparison of pML and pML-2 amino acid sequences shows the latter form is identical except that the tetrapeptide QLPP corresponding to residues 111-114 inclusive have been deleted (see **Fig. 22** [SEQ ID NOS: 18 & 21]). The four amino acid deletions observed in murine, human and porcine ML cDNA occur at precisely the same position within the predicted proteins

EXAMPLE 14

CMK Assay for Thrombopoietin (TPO) Induction of Platelet Antigen $GPII_bIII_a$ Expression

CMK cells are maintained in RMPI 1640 medium (Sigma) supplemented with 10% fetal bovine serum and 10mM glutamine. In preparation for the assay, the cells are harvested, washed and resuspended at 5×10^5 cells/ml in serum-free GIF medium supplemented with 5mg/l bovine insulin, 10mg/l apo-transferrin, 1 X trace elements. In a 96-well flat-bottom plate, the TPO standard or experimental samples are added to each well at appropriate dilutions in 100 µl volumes. 100 µl of the CMK cell suspension is added to each well and the plates are incubated at 37°C, in a 5% CO_2 incubator for 48 hours. After incubation, the plates are spun at 1000rpm at 4°C for five minutes. Supernatants are discarded and 100 µl of the FITC-conjugated $GPII_bIII_a$ monoclonal 2D2 antibody is added to each well. Following incubation at 4°C for 1 hour, plates are spun again at 1000rpm for five minutes. The supernatants containing unbound antibody are discarded and 200 µl of 0.1% BSA-PBS wash is added to each well. The 0.1% BSA-PBS wash step is repeated three times. Cells are then analyzed on a FASCAN using standard one parameter analysis measuring relative fluorescence intensity.

EXAMPLE 15

DAMI Assay for Thrombopoietin (TPO) by Measuring Endomitotic Activity of DAMI Cells on 96-well Microtiter Plates

DAMI cells are maintained in IMDM + 10% horse serum (Gibco) supplemented with 10mM glutamine, 100ng/ml Penicillin G, and 50 μg/ml streptomycin. In preparation for the assay, the cells are harvested, washed, and resuspended at 1×10^6 cells/ml in IMDM + 1% horse serum. In a 96-well round-bottom plate, 100 μl of the TPO standard or experimental samples is added to DAMI cell suspension. Cells are then incubated for 48 hours at 37°C in a 5% CO_2 incubator. After incubation, plates are spun in a Sorvall 6000B centrifuge at 1000rpm for five minutes at 4°C. Supernatants are discarded and 200 μl of PBS-0.1% BSA wash step is repeated. Cells are fixed by the addition of 200 μl ice-cold 70% Ethanol-PBS and resuspended by aspiration. After incubation at 4°C for 15 minutes, the plates are spun at 2000 rpm for five minutes and 150 μl of 1mg/ml RNAse containing 0.1mg/ml propidium iodide and 0.05% Tween-20 is added to each well. Following a one hour incubation at 37°C the changes in DNA content are measured by flow cytometry. Polyploidy is measured and quantitated as follows:

$$\text{Normalized Polyploid Ratio (NPR)} = \frac{(\%\text{Cells in} > G2{+}M/\%\text{Cells in} < G2{+}M) \text{ with TPO}}{(\%\text{Cells in} > G2{+}M/\%\text{Cells in} < G2{+}M) \text{ in control}}$$

EXAMPLE 16

Thrombopoietin (TPO) In Vivo Assay
(Mouse Platelet Rebound Assay)
In Vivo Assay for ^{35}S Determination of Platelet Production

C57BL6 mice (obtained from Charles River) are injected intraperitoneally (IP) with 1 ml goat anti-mouse platelet serum (6 amps) on day 1 to produce thrombocytopenia. On days 5 and 6, mice are given two IP injections of the factor or PBS as the control. On day 7, thirty μCi of $Na_2{}^{35}SO_4$ in 0.1 ml saline are injected intravenously and the percent ^{35}S incorporation of the injected dose into circulating platelets is measured in blood samples obtained from treated and control mice. Platelet counts and leukocyte counts are made at the same time from blood obtained from the retro-orbital sinus.

EXAMPLE 17

KIRA ELISA for Thrombopoietin (TPO) by Measuring Phosphorylation of the mpl-Rse.gD Chimeric Receptor

The human *mpl* receptor has been disclosed by Vigon *et al.*, *PNAS, USA* 89:5640-5644 (1992). A chimeric receptor comprising the extracellular domain (ECD) of the *mpl* receptor and the transmembrane (TM) and intracellular domain (ICD) of *Rse* (Mark *et al.*, *J. of Biol. Chem.* **269(14)**:10720-10728 [1994]) with a carboxyl-terminal flag polypeptide (*i.e.* Rse.gD) was made for use in the KIRA ELISA described herein. See **Fig. 30** and **31** for a diagrammatic description of the assay.

(a) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky *et al.*, Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20° C.

(b) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH)

(c) Ligand

The *mpl* ligand was prepared by the recombinant techniques described herein. The purified *mpl* ligand was stored at 4 °C as a stock solution

(d) Preparation of Rse.gD nucleic acid

Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human Rse and add an additional 21 amino acids containing an epitope for the antibody 5B6 and a stop codon. **Table 10** presents the final sequence of the synthetic portion of the fusion gene.

TABLE 10

Synthetic Double Stranded Portion of Human Rse Fusion Gene

coding strand: 5'-TGCAGCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGA TGGCTG ATCCAAATCGATTCCGCGGCAAAGATCTTCCGGTCCTGTAGAAGCT-3'	(SEQ ID NO: 59)
noncoding (anti-sense) strand: 5'-AGCTTCTACAGGACCGGAAGATCTTTGCCGCGGAATCGATTTGGATCAGCCA TCTTG AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTTGCTGCA-3'	(SEQ ID NO: 60)

The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark *et al.*, Journal of Biological Chemistry **269(14)**:10720-10728 [1994]) and HindIII sites in the polylinker of the expression vector pSVI7.ID.LL (See **Fig. 32 A-L**; SEQ ID NO: 22) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron splice sites, followed by sequence that encodes the Rse.gD. The full length (non-spliced) message contains DHFR as the first open reading frame and therefore generates DHFR protein to allow selection of stable transformants.

(e) Preparation of mpl-Rse.gD nucleic acid

The expression plasmid pSV.ID.Rse.gD produced as described above was modified to produce plasmid pSV.ID.M.tmRd6 which contained the coding sequences of the ECD of human *mpl* (amino acids 1-491) fused to the transmembrane domain and intracellular domain of Rse.gD (amino acids 429-911). Synthetic oligonucleotides were used to join the coding sequence of a portion of the extracellular domain of human *mpl* to a portion of the Rse coding sequence in a two step PCR cloning reaction as described by Mark *et al.*, *J. Biol Chem* **267**:26166-26171 (1992). Primers used for the first PCR reaction were M1

(5'-TCTCGCTACCGTTTACAG-3')

(SEQ ID NO: 61)

and M2

(5'-CAGGTACCCACCAGGCGGTCTCGGT-3')

(SEQ ID NO: 62)

with a *mpl* cDNA template and R1

(5'-GGGCCATGACACTGTCAA-3')

(SEQ ID NO: 63)

and R2

(5'-GACCGCCACCGAGACCGCCTGGTGGGTACCTGTGGTCCTT-3')

(SEQ ID NO: 64)

with a Rse cDNA template. The PvuII-SmaI portion of this fusion junction was used for the construction of the full-length chimeric receptor.

(f) Cell transformation

DP12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with pSV.ID.M.tmRd6 which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20µl 1/10 Tris EDTA. Then, 10µg of DNA was incubated with 10^7 CHO DP12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 400 volts

and 330µl. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

(g) Selection of transformed cells for use in the KIRA ELISA

Clones expressing *MPL*/Rse.gD were identified by western-blotting of whole cell lysates post-fractionation by SDS-PAGE using the antibody 5B6 which detects the gD epitope tag.

(h) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine

(i) KIRA ELISA

Mpl-Rse.gD transformed DP12 CHO cells were seeded (3×10^4 per well) in the wells of a flat-bottom-96 well culture plate in 100 µl media and cultured overnight at 37 °C in 5% CO_2. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50µl of media containing either experimental samples or 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml *mpl* ligand was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the chimeric receptors, 100 µl of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 µM leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

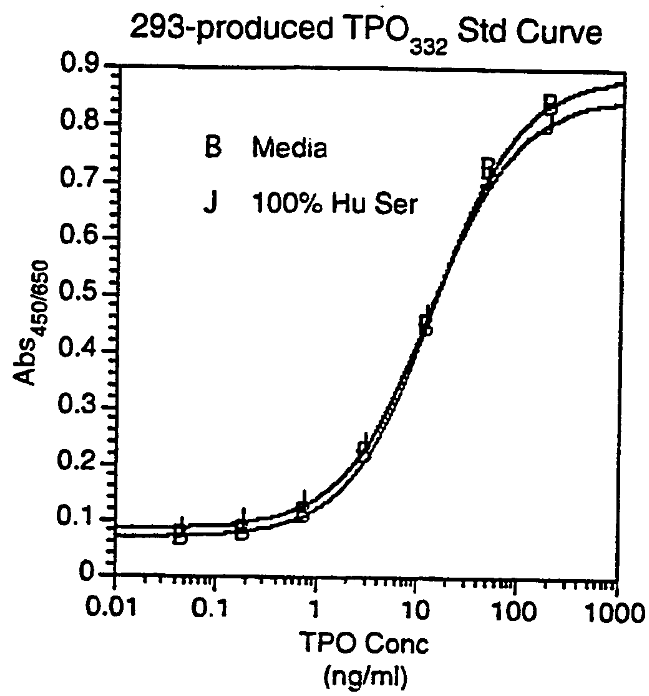
While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (5.0 µg/ml in 50 mM carbonate buffer, pH 9.6, 100 µl/well) was decanted, tamped on a paper towel and blocked with 150 µl/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized MPL/Rse.gD from the cell-culture microtiter well was transferred (85 µl/well) to anti-gD 5B6 coated and blocked ELISA well and

was incubated for 2 h at room temperature with gentle agitation. The unbound *mpl*-Rse.gD was removed by washing with wash buffer and 100 µl of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:18000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.* 56 ng/ml was added to each well. After incubation for 2 hr at room temperature the plate was washed and 100 µl of horseradish peroxidase (HRPO)-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:60000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 µl freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 µl/well 1.0 M H_3PO_4. The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve was generated by stimulating dp12.trkA,B or C.gD cells with 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml *mpl* ligand and presented as ng/ml TPO vs. mean $ABS_{450/650}$ ± sd using the DeltaSoft program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of ng/ml TPO activity.

The *mpl*-ligand was found to be able to activate the *mpl*-Rse.gD chimeric receptor in a concentration-dependent and ligand-specific manner. Further, the *mpl*-Rse.gD KIRA–ELISA was found to be tolerant of up to 100% human serum (shown) or 100% plasma (not shown), allowing the assay to be used to readily screen patient and pK samples.



Summary of TPO EC50's

TPO Form (cells)	EC50 (wt/vol)	EC50 (molarity)
Hu TPO 332 (293)	2.56 ng/ml	67.4 pM
Mu TPO 332 (293)	3.69 ng/ml	97.1 pM
Hu TPO 153 (293)	~41 ng/ml	~1.08 nM
Hu TPO 155 (*E. coli*)	0.44 ng/ml	11.6 pM
Hu TPO 153met (*E. coli*)	0.829 ng/ml	21.8 pM

EXAMPLE 18

Receptor Based ELISA for Thrombopoietin (TPO)

ELISA plates were coated with rabbit $F(ab')_2$ anti-human IgG (Fc) in pH 9.6 carbonate buffer at 4°C overnight. Plates were blocked with 0.5 % bovine serum albumin in PBS at room temperature for one hour. Fermenter harvest containing the chimeric receptor, *mpl*-IgG, was added to the plates and incubated for 2 hours. Twofold serial dilutions (0.39-25 ng/ml) of the standard (TPO_{332} produced in 293 cells with the concentration determined by quantitative amino acid analysis) and serially diluted samples in 0.5% bovine serum albumin, 0.05% tween 20 were added to the

plates and incubated for 2 hours. Bound TPO was detected with protein A purified, biotinylated rabbit antibodies to TPO_{155} which was produced in E. coli (1 hour incubation), followed by streptavidin-peroxidase (30 min. incubation) and 3,3',5,5'-tetramethyl benzidine as the substrate. The absorbance was read at 450 nm. Plates were washed between steps. For data analysis, the standard curve is fitted using a four-parameter curve fitting program by Kaleidagraph. Concentrations of the samples were calculated from the standard curve.

EXAMPLE 19

Expression and Purification of TPO from 293 Cells

1. *Preperation of 293 Cell Expression Vectors*

A cDNA corresponding to the TPO entire open reading frame was obtained by PCR using the following oligonucleotides as primers:

TABLE 11

293 PCR Primers

Cla.FL.F: 5' ATC GAT ATC GAT CAG CCA GAC ACC CCG GCC AG 3'	(SEQ ID NO: 65)
hmpll-R: 5' GCT AGC TCT AGA CAG GGA AGG GAG CTG TAC ATG AGA 3'	(SEQ ID NO: 48)

PRK5-h*mpl* I (described in **Example 9**) was used as template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C). The PCR product was purified and cloned between the restriction sites ClaI and XbaI of the plasmid pRK5tkneo, a pRK5 derived vector modified to express a neomycin resistance gene under the control of the thymidine kinase promote, to obtain the vector pRK5tkneo.ORF. A second construct corresponding to the epo homologous domain was generated the same way but using Cla.FL.F as forward primer and the following reverse primer:

Arg.STOP.Xba: 5' TCT AGA TCT AGA TCA CCT GAC GCA GAG GGT GGA CC 3' (SEQ ID NO: 66)

The final construct is called pRK5-tkneoEPO-D. The sequence of both constructs was verified as described in **Example 7**.

2. *Transfection of Human Embryonic Kidney cells*

These 2 constructs were transfected into Human Embryonic Kidney cells by the $CaPO_4$ method as described in **Example 9**. 24 hours after transfection selection of neomycin resistant clones was started in the presence of 0.4 mg/ml G418.10 to 15

days later individual colonies were transferred to 96 well plates and allowed to grow to confluency. Expression of ML_{153} or ML_{332} in the conditioned media from these clones was assessed using the Ba/F3-*mpl* proliferation assay (described in **Example I**).

3. Purification of $rhML_{332}$

293-$rhML_{332}$ conditioned media was applied to a Blue-Sepharose (pharmacia) column that was equilibrated in 10mM sodium phosphate pH 7.4 (buffer A). The column was subsequently washed with 10 column volumes each of buffer A and buffer A containing 2M urea. The column was then eluted with buffer A containing 2M urea and 1M NaCl. The Blue-Sepharose elution pool was then directly applied to a WGA-Sepharose column equilibrated in buffer A. The WGA-Sepharose column was then washed with 10 column volumes of buffer A containing 2M urea and 1 M NaCl and eluted with the same buffer containing 0.5M N-acetyl-D-glucosamine. The WGA-Sepharose eluate was applied to a C4-HPLC column (Synchrom, Inc.) equilibrated in 0.1% TFA. The C4-HPLC column was eluted with discontinuous propanol gradient (0-25%, 25-35%, 35-70%). $rhML_{332}$ was found to elute in the 28-30% propanol region of the gradient By SDS-PAGE the purified $rhML_{332}$ migrates as a broad band in the 68-80 kDa region of the gel(see **Figure 15**)

4 Purification of $rhML_{153}$

293-$rhML_{153}$ conditioned media was resolved on Blue-Sepharose as described for $rhML_{332}$ The Blue Sepharose eluate was applied directly to a *mpl*-affinity column as described above. $RhML_{153}$ eluted from the *mpl*-affinity column was purified to homogeneity using a C4-HPLC column run under the same conditions as described for $rhML_{332}$. By SDS-PAGE the purified $rhML_{153}$ resolves into 2 major and 2 minor bands with Mr of ~18,000-21,000(see **Figure 15**)

EXAMPLE 20

Expression and Purification of TPO from CHO

1. Description of CHO Expression Vectors

The expression vectors used in the electroporation protocols described below have been designated:

pSVI5.ID.LL.MLORF (full length or $hTPO_{332}$), and

pSVI5.ID.LL.MLEPO-D (truncated or $hTPO_{153}$).

The pertinent features of these plasmids are presented in **Fig. 23** and **24.**

2. Preperation of CHO Expression Vectors

A cDNA corresponding to the hTPO entire open reading frame was obtained by PCR using the oligonucleotide primers of **Table 12**.

TABLE 12

CHO Expression Vector PCR Primers

Cla.FL.F2	5' ATC GAT ATC GAT AGC CAG ACA CCC CGG CCA G 3'	(SEQ ID NO: 47)
ORF. Sal	5' AGT CGA CGT CGA CGT CGG CAG TGT CTG AGA ACC 3'	(SEQ ID NO: 67)

PRK5-h*mpl* I (described in **Example 7** and **9**) was used as template for the reaction in the presence of pfu DNA polymerase (Stratagene). Initial denaturation was for 7 min. at 94°C followed by 25 cycles of amplification (1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C). Final extension was for 15 min. at 72°C). The PCR product was purified and cloned between the restriction sites ClaI and SalI of the plasmid pSVI5.ID.LL to obtain the vector pSVI5.ID.LL.MLORF. A second construct corresponding to the EPO homologous domain was generated the same way but using Cla.FL.F2 as forward primer and the following reverse primer:

EPOD.Sal 5' AGT CGA CGT CGA CTC ACC TGA CGC AGA GGG TGG ACC 3' (SEQ ID NO: 68)

The final construct is called pSVI5.ID.LL.MLEPO-D. The sequence of both constructs was verified as described in **Example 7** and **9**.

In essence, the coding sequences for the full length and truncated ligand were introduced into the multiple cloning site of the CHO expression vector pSVI5.ID.LL This vector contains the SV40 early promoter/enhancer region, a modified splice unit containing the mouse DHFR cDNA, a multiple cloning site for the introduction of the gene of interest (in this case the TPO sequences described) an SV40 polyadenylation signal and origin of replication and the beta-lactamase gene for plasmid selection and amplification in bacteria.

3. Methodology for Establishing Stable CHO Cell Lines Expressing Recombinant Human TPO_{332} and TPO_{153}

a. Description of CHO parent cell line

The host CHO (Chinese Hamster Ovary) cell line used for the expression of the TPO molecules described herein is known as CHO-DP12 (see EP 307,247 published 15 March 1989). This mammalian cell line was clonally selected from a transfection of the parent line (CHO-K1 DUX-B11(DHFR-)- obtained from Dr. Frank Lee of Stanford University with the permission of Dr.L. Chasin) with a vector expressing preproinsulin to obtain clones with reduced insulin requirements. These cells are also DHFR minus and clones can be selected for the presence of DHFR cDNA vector sequences by growth on medium devoid of nucleoside supplements (glycine, hypoxanthine, and

thymidine). This selection system for stably expressing CHO cell lines is commonly used.

b. Transfection method (electroporation)

TPO_{332} and TPO_{153} expressing cell lines were generated by transfecting DP12 cells via electroporation (see *e.g.* Andreason, G.L. *J. Tiss. Cult. Meth.*, 15,56 [1993]) with linearized pSVI5.ID.LL.MLORF or pSVI5.ID.LL.MLEPO-D plasmids respectively. Three (3) restriction enzyme reaction mixtures were set up for each plasmid cutting; 10μg, 25μg and 50μg of the vector with the enzyme NOTI by standard molecular biology methods. This restriction site is found only once in the vector in the linearization region 3' and outside the TPO ligand transcription units (see **Fig. 23**). The 100μl reactions were set up for overnight incubation at 37 degrees. The next day the mixes were phenol-chloroform-isoamyl alcohol (50:49:1) extracted one time and ethanol precipitated on dry ice for approximately one hour. The precipitate was then collected by a 15 minute microcentrifugation and dried. The linearized DNA was resuspended into 50μl of Ham's DMEM-F12 1:1 medium supplemented with standard antibiotics and 2mM glutamine.

Suspension growing DP12 cells were collected, washed one time in the medium described for resuspending the DNA and finally resuspended in the same medium at a concentration of 10^7 cells per 750μl Aliquots of cells (750μl) and each linearized DNA mix were incubated together at room temperature for one hour and then transferred to a BRL electroporation chamber. Each reaction mix was then electroporated in a standard BRL electroporation apparatus at 350 volts set at 330μF and low capacitance. After electroporation, the cells were allowed to sit in the apparatus for 5 minutes and then on ice for an additional 10 minute incubation period. The electroporated cells were transferred to 60mm cell culture dishes containing 5 ml of standard, complete growth medium for CHO cells (High glucose DMEM-F12 50:50 without glycine supplemented with 1X GHT, 2mM glutamine, and 5% fetal calf serum) and grown overnight in a 5% CO_2 cell culture incubator.

c. Selection and screening method

The next day, cells were trypsinized off the plates by standard methods and transferred to 150mm tissue culture dishes containing DHFR selective medium (Ham's DMEM-F12, 1:1medium described above supplemented with either 2% or 5% dialyzed fetal calf serum but devoid of glycine, hypoxanthine and thymidine this is the standard DHFR selection medium we use). Cells from each 60mm dish were subsequently replated into 5 /150 mm dishes. Cells were then incubated for 10 to 15 days(with one medium change) at 37 degrees/5% CO_2 until clones began to appear and reached sizes amenable to transfer to 96 well dishes. Over a period of 4-5 days, cell lines were transferred to 96 well dishes using sterile yellow tips on a pipettman set at

50ml. The cells were allowed to grow to confluency (usually 3-5 days) and then the trays were trypsinized and 2 copies of the original tray were reproduced. Two of these copies were short term stored in the freezer with cells in each well diluted into 50µl of 10%FCS in DMSO. 5 day conditioned serum free medium samples were assayed from confluent wells in the third tray for TPO expression via the Ba/F cell based activity assay. The highest expressing clones based on this assay were revived from storage and scaled up to 2 confluent 150mm T-flasks for transfer to the cell culture group for suspension adaptation, re-assay and banking.

d. Amplification Protocol

Several of the highest titer cell lines from the selection described above were subsequently put through a standard methotrexate amplification regime to generate higher titer clones. CHO cell clones are expanded and plated in 10cm dishes at 4 concentrations of methotrexate (*i.e.*. 50nM, 100nM, 200nM and 400nM) at two or three cell numbers (105, 5x105, and 106 cells per dish). These cultures are then incubated at 37 degree/5% CO_2 until clones are established and amenable to transfer to 96 well dishes for further assay. Several high titer clones from this selection were again subjected to greater concentrations of methotrexate (*i.e.* 600nM, 800 nM, 1000nM and 1200nM) and as before resistant clones are allowed to establish and then transferred to 96 well dishes and assayed.

4. Culturing Stable CHO Cell Lines Expressing Recombinant Human TPO_{332} and TPO_{153}

Banked cells are thawed and the cell population is expanded by standard cell growth methods in either serum free or serum containing medium. After expansion to sufficient cell density, cells are washed to remove spent cell culture media. Cells are then cultured by any standard method including; batch, fed-batch or continuous culture at 25-40 °C, neutral pH, with a dissolved O_2 content of at least 5% until the constitutively secreted TPO is accumulated. Cell culture fluid is then separated from the cells by mechanical means such as centrifugation.

5 Purification of Recombinant Human TPO from CHO Culture Fluids

Harvested cell culture fluid (HCCF) is directly applied to a Blue Sepharose 6 Fast Flow column (Phamacia) equilibrated in 0.01M Na Phosphate pH7.4, 0.15M NaCl at a ratio of approximately 100L of HCCF per liter of resin and at a linear flow rate of approximately 300 ml/hr/cm^2. The column is then washed with 3 to 5 column volumes of equilibration buffer followed by 3 to 5 column volumes of 0.01M Na Phosphate pH7.4, 2.0M urea. The TPO is then eluted with 3 to 5 column volumes of 0.01M Na Phosphate pH7.4, 2.0M urea, 1.0M NaCl.

The Blue Sepharose Pool containing TPO is then applied to a Wheat Germ Lectin Sepharose 6MB column (Pharmacia) equilibrated in 0.01M Na Phosphate pH7.4,

2.0M urea, and 1.0M NaCl at a ratio of from 8 to 16 ml of Blue Sepharose Pool per ml of resin at flow rate of approximately 50 ml/hr/cm^2. The column is then washed with 2 to 3 column volumes of equilibration buffer. The TPO is then eluted with 2 to 5 column volumes of 0.01M Na Phosphate pH7.4, 2.0M urea, 0.5M N-acetyl-D-glucosamine.

The Wheat Germ Lectin Pool is then adjusted to a final concentration of 0.04% $C_{12}E_8$ and 0.1% trifluroacetic acid (TFA). The resulting pool is applied to a C4 reverse phase column (Vydac 214TP1022) equilibrated in 0.1% TFA, 0.04% $C_{12}E_8$ at a load of approximately 0.2 to 0.5 mg protein per ml of resin at a flow rate of 157 ml/hr/cm^2.

The protein is eluted in a two phase linear gradient of acetonitrile containing 0.1% TFA, 0.04% $C_{12}E_8$. The first phase is composed of a linear gradient from 0 to 30% acetonitrile in 15 minutes. The second phase is composed of a linear gradient from 30 to 60% acetonitrile in 60 minutes. The TPO elutes at approximately 50% acetonitrile. A pool is made on the basis of SDS-PAGE.

The C4 Pool is then diluted with 2 volumes of 0.01M Na Phosphate pH7.4, 0.15M NaCl and diafilitered versus approximately 6 volumes of 0.01M Na Phosphate pH7.4, 0.15M NaCl on an Amicon YM or like ultrafiltration membrane having a 10,000 to 30,000 Dalton molecular weight cut-off. The resulting diafiltrate may be then directly processed or further concentrated by ultrafiltration. The diafiltrate/concentrate is adjusted to a final concentration of 0.01% Tween-80.

All or a portion of the diafiltrate/concentrate equivalent to 2 to 5% of the calculated column volume is then applied to a Sephacryl S-300 HR column (Pharmacia) equilibrated in 0.01M Na Phosphate pH7.4, 0.15M NaCl, 0.01% Tween-80 and chromatographed at a flow rate of approximately 17 ml/hr/cm^2. The TPO containing fractions which are free of aggregate and proteolytic degradation products are pooled on the basis of SDS-PAGE. The resulting pool is filtered on a 0.22μ filter, Millex-GV or like, and stored at 2-8°C.

EXAMPLE 21

Transformation and Induction of TPO Protein Synthesis in E. coli

1. *Construction of E. coli TPO expression vectors*

The plasmids pMP21, pMP151, pMP41, pMP57 and pMP202 are all designed to express the first 155 amino acids of TPO downstream of a small leader which varies among the different constructs. The leaders provide primarily for high level translation initiation and rapid purification. The plasmids pMP210-1, -T8, -21, -22, -24, -25 are designed to express the first 153 amino acids of TPO downstream of an initiation methionine and differ only in the codon usage for the first 6 amino acids

of TPO, while the plasmid pMP251 is a derivative of pMP210-1 in which the carboxy terminal end of TPO is extended by two amino acids. All of the above plasmids will produce high levels of intracellular expression of TPO in E. coli upon induction of the tryptophan promoter (Yansura, D. G. *et. al. Methods in Enzymology* (Goeddel, D. V., Ed.) **185**:54-60, Academic Press, San Diego [1990]). The plasmids pMP1 and pMP172 are intermediates in the construction of the above TPO intracellular expression plasmids.

(a) Plasmid pMP1

The plasmid pMP1 is a secretion vector for the first 155 amino acids of TPO, and was constructed by ligating together 5 fragments of DNA as shown in **Fig. 33**. The first of these was the vector pPho21 in which the small MluI-BamHI fragment had been removed. pPho21 is a derivative of phGH1 (Chang, C. N. *et. al., Gene* 55:189-196 [1987]) in which the human growth hormone gene has been replaced with the E. coli phoA gene, and a MluI restriction site has been engineered into the coding sequence for the STII signal sequence at amino acids 20-21.

The next two fragments, a 258 base pair HinfI-PstI piece of DNA from pRK5-h*mpl* I **(Example 9)** encoding TPO amino acids 19-103, and the following synthetic DNA encoding amino acids 1-18

5'-CGCGTATGCCAGCCCGGCTCCTCCTGCTTGTGACCTCCGAGTCCTCAGTAAACTGCTTCG
TG

ATACGGTCGGGCCGAGGAGGACGAACACTGGAGGCTCAGGAGTCATTTGACGAAGC
ACTGA-5'

(SEQ ID NO: 69)

(SEQ ID NO: 70)

were preligated with T4-DNA ligase, and second cut with PstI. The fourth was a 152 base pair PstI-HaeIII fragment from pRK5hmplI encoding amino acids 104-155 of TPO. The last was a 412 base pair StuI-BamHI fragment from pdh108 containing the lambda to transcriptional terminator as previously described (Scholtissek, S. *et. al., NAR* 15:3185 [1987]).

(b) Plasmid pMP21

The plasmid pMP21 is designed to express the first 155 amino acids of TPO with the aid of a 13 amino acid leader comprising part of the STII signal sequence. It was constructed by ligating together three (3) DNA fragments as shown in **Fig. 34**, the first of these being the vector pVEG31 in which the small XbaI-SphI fragment had been removed. The vector pVEG31 is a derivative of pHGH207-1 (de Boer, H. A. *et. al.* , in *Promoter Structure and Function* (Rodriguez, R. L. and Chamberlain, M. J. , Ed), 462, Praeger, New York [1982]) in which the human growth hormone gene has been

replaced by the gene for vascular endothelial growth factor (this identical vector fragment can be obtained from this latter plasmid).

The second part in the ligation was a synthetic DNA duplex with the following sequence:

5'-CTAGAATTATGAAAAAGAATATCGCATTTCTTCTTAA
TTAATACTTTTTCTTATAGCGTAAAGAAGAATTGCGC-5'
(SEQ ID NO: 71)
(SEQ ID NO. 72)

The last piece was a 1072 base pair MluI-SphI fragment from pMP1 encoding 155 amino acids of TPO.

(c) Plasmid pMP151

The plasmid pMP151 is designed to express the first 155 amino acids of TPO downstream of a leader comprising 7 amino acids of the STII signal sequence, 8 histidines, and a factor Xa cleavage site As shown in **Fig. 35**, pMP151 was constructed by ligating together three DNA fragments, the first of these being the previously described vector pVEG31 from which the small XbaI-SphI fragment had been removed The second was a synthetic DNA duplex with the following sequence

5'-CTAGAATTATGAAAAAGAATATCGCATTTCATCACCATCACCATCACCATCACATCGAAG GTCGTAGCC
TTAATACTTTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTTC CAGCAT-5
(SEQ ID NO 73)
(SEQ ID NO 74)

The last was a 1064 base pair BglI-SphI fragment from pMP11 encoding 154 amino acids of TPO. The plasmid pMP11 is identical to pMP1 with the exception of a few codon changes in the STII signal sequence(this fragment can be obtained from pMP1).

(d) Plasmid pMP202

The plasmid pMP202 is very similar to the expression vector pMP151 with the exception that the factor Xa cleavage site in the leader has been replaced with a thrombin cleavage site. As shown in **Fig. 36**, pMP202 was constructed by ligating together three DNA fragments. The first of these was the previously described pVEG31 in which the small XbaI-SphI fragment had been removed. The second was a synthetic DNA duplex with the following sequence

5'-CTAGAATTATGAAAAAGAATATCGCATTTCATCACCATCACCATCACCATCACATCGAA CCACGTAGCC

TTAATACTTTTTCTTATAGCGTAAAGTAGTGGTAGTGGTAGTGGTAGTGTAGCTT

GGTGCAT-5'

(SEQ ID NO: 75)

(SEQ ID NO: 76)

The last piece was a 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

(e) Plasmid pMP172

The plasmid pMP172 is a secretion vector for the first 153 amino acids of TPO, and is an intermediate for the construction of pMP210. As shown in **Fig. 37**, pMP172 was prepared by ligating together three DNA fragments, the first of which was the vector pLS32lamB in which the small EcoRI-HindIII section had been removed. The second was a 946 base pair EcoRI-HgaI fragment from the previously described plasmid pMP11. The last piece was a synthetic DNA duplex with the following sequence.

5'-TCCACCCTCTGCGTCAGGT (SEQ ID NO: 77)

GGAGACGCAGTCCATCGA-5' (SEQ ID NO: 78)

(f) Plasmid pMP210

The plasmid pMP210 is designed to express the first 153 amino acids of TPO after a translational initiation methionine This plasmid was actually made as a bank of plasmids in which the first 6 codons of TPO were randomized in the third position of each codon, and was constructed as shown in **Fig. 38** by the ligation of three DNA fragments. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed The second was a synthetic DNA dupex shown below treated first with DNA polymeraseI (Klenow) followed by digestion with XbaI and HinfI, and encoding the initation methionine and the randomized first 6 codons of TPO

5'-GCAGCAGTTCTAGAATTATGTCNCCNGCNCCNCCNGCNTGTGACCTCCGA

ACACTGGAGGCT

GTTCTCAGTAAA (SEQ ID NO: 79)

CAAGAGTCATTTGACGAAGCACTGAGGGTACAGGAAG-5' (SEQ ID NO: 80)

The third was a 890 base pair HinfI-SphI fragment from pMP172 encoding amino acids 19-153 of TPO.

The plasmid pMP210 bank of approximately 3700 clones was retransformed onto high tetracycline (50 μg/ml) LB plates to select out high translational initiation clones (Yansura, D. G. *et. al., Methods: A Companion to Methods in Enzymology* 4:151-158 [1992]). Of the 8 colonies which came up on high tetracycline plates, five of the

best in terms of TPO expression were subject to DNA sequencing and the results are shown in **Fig. 39** (SEQ ID NOS: 23, 24, 25, 26, 27 and 28).

(g) Plasmid pMP41

The plasmid pMP41 is designed to express the first 155 amino acids of TPO fused to a leader consisting of 7 amino acids of the STII signal sequence followed by a factor Xa cleavage site. The plasmid was constructed as shown in **Fig. 40** by ligating together three pieces of DNA, the first of which was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following synthetic DNA duplex:

5'-CTAGAATTATGAAAAAGAATATCGCATTTATCGAAGGTCGTAGCC (SEQ ID NO: 81)
TTAATACTTTTTCTTATAGCGTAAATAGCTTCCAGCAT-5' (SEQ ID NO: 82)

The last piece of the ligation was the 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

(h) Plasmid pMP57

The plasmid pMP57 expresses the first 155 amino acids of TPO downstream of a leader consisting of 9 amino acids of the STII signal sequence and the dibasic site Lys-Arg. This dibasic site provides for a means of removing the leader with the protease ArgC. This plasmid was constructed as shown in **Fig. 41** by ligating together three DNA pieces. The first of these was the previously described vector pVEG31 in which the small XbaI-SphI fragment had been removed. The second was the following synthetic DNA duplex

5'-CTAGAATTATGAAAAAGAATATCGCATTTCTTCTTAAACGTAGCC (SEQ ID NO: 83)
TTAATACTTTTTCTTATAGCGTAAAGAAGAATTTGCAT-5' (SEQ ID NO: 84)

The last part of the ligation was the 1064 base pair BglI-SphI fragment from the previously described plasmid pMP11.

(i) Plasmid pMP251

The plasmid pMP251 is a derivative of pMP210-1 in which two additional amino acids of TPO are included on the carboxy terminal end. As shown in **Fig.42**, this plasmid was constructed by ligating together two pieces of DNA, the first of these being the previously described pMP21 in which the small XbaI-ApaI fragment had been removed. The second part of the ligation was a 316 base pair XbaI-ApaI fragment from pMP210-1.

2. Transformation and Induction of E. coli with TPO expression vectors

The above TPO expression plasmids were used to transform the *E. coli* strain 44C6 (w3110 tonA$_\Delta$ rpoH$_{ts}$ lon$_\Delta$ clpP$_\Delta$ galE) using the $CaCl_2$ heat shock method (Mandel, M. *et al.*, *J. Mol. Biol.*, 53:159-162, [1970]). The transformed cells were grown first at 37°C in LB media containing 50 µg/ml carbenicillin until the optical density (600nm) of the culture reached approximately 2-3. The LB culture was then

diluted 20x into M9 media containing 0.49% casamino acids (w/v) and 50 μg/ml carbenicillin. After growth with aeration at 30°C for 1 hour, indole-3-acrylic acid was added to a final concentration of 50 μg/ml. The culture was then allowed to continue growing at 30°C with aeration for another 15 hours at which time the cells were harvested by centrifugation.

EXAMPLE 22

Production of Biologically Active TPO (Met^{-1} 1-153) in E. coli

The procedures given below for production of biologically active, refolded TPO (met^{-1} 1-153) can be applied in analogy for the recovery of other TPO variants including N and C terminal extended forms (see **Example 23**).

A Recovery of non-soluble TPO (Met^{-1} 1-153)

E. coli cells expressing TPO (Met^{-1} 1-153) encoded by the plasmid pMP210-1 are fermented as described above. Typically, about 100g of cells are resuspended in 1 L (10 volumes) of cell disruption buffer (10 mM Tris, 5 mM EDTA, pH 8) with a Polytron homogenizer and the cells centrifuged at 5000 x g for 30 minutes. The washed cell pellet is again resuspended in 1 L cell disruption buffer with the Polytron homogenizer and the cell suspension is passed through an LH Cell Disrupter (LH Inceltech, Inc.) or through a Microfluidizer (Microfluidics International) according to the manufactures' instructions. The suspension is centrifuged at 5,000g for 30 min. and resuspended and centrifuged a second time to make a washed refractile body pellet. The washed pellet is used immediately or stored frozen at -70°C.

B. Solubilization and purification of monomeric TPO (Met^{-1} 1-153)

The pellet from above is resuspended in 5 volumes by weight of 20 mM Tris, pH 8, with 6-8 M guanidine and 25 mM DTT (dithiothreitol) and stirred for 1-3 hr., or overnight, at 4°C to effect solubilization of the TPO protein. High concentrations of urea (6-8M) are also useful but generally result in lower yields compared to guanidine. After solubilization, the solution is centrifuged at 30,000 x g for 30 min. to produce a clear supernatant containing denatured, monomeric TPO protein. The supernatant is then chromatographed on a Superdex 200 gel filtration column (Pharmacia, 2.6 x 60 cm) at a flow rate of 2 ml/min. and the protein eluted with 20 mM Na phosphate, pH 6.0, with 10 mM DTT Fractions containing monomeric, denatured TPO protein eluting between 160 and 200 ml are pooled. The TPO protein is further purified on a semi-preparative C4 reversed phase column (2 x 20 cm VYDAC). The sample is applied at 5 ml/min. to a column equilibrated in 0.1% TFA(trifluoroacetic acid) with 30% acetonitrile. The protein is eluted with a linear gradient of acetonitrile (30-60% in 60 min.). The purified reduced protein elutes at

approximately 50% acetonitrile. This material is used for refolding to obtain biologically active TPO variant.

C. *Generation of biologically active TPO (Met^{-1} 1-153)*

Approximately 20 mg of monomeric, reduced and denatured TPO protein in 40 ml 0.1% TFA/50% acetonitrile is diluted into 360 ml of refolding buffer containing optimally the following reagents:

50 mM Tris
0.3 M NaCl
5 mM EDTA
2% CHAPS detergent
25% glycerol
5 mM oxidized glutathione
1 mM reduced glutathione
pH adjusted to 8.3

After mixing, the refolding buffer is gently stirred at 4°C for 12-48 hr to effect maximal refolding yields of the correct disulfide-bonded form of TPO (see below). The solution is then acidified with TFA to a final concentration of 0.2%, filtered through a 0.45 or 0.22 micron filter, and 1/10 volume of acetonitrile added. This solution is then pumped directly onto a C4 reversed phase column and the purified, refolded TPO (Met^{-1} 1-153) eluted with the same gradient program as above. Refolded, biologically active TPO is eluted at approximately 45% acetonitrile under these conditions. Improper disulfide-bonded versions of TPO are eluted earlier. The final purified TPO (Met^{-1} 1-153) is greater than 95% pure as assessed by SDS gels and analytical C4 reversed phase chromatography. For animal studies, the C4 purified material was dialyzed into physiologically compatible buffers. Isotonic buffers (10 mM Na acetate, pH 5.5, 10 mM Na succinate, pH 5.5 or 10 mM Na phosphate, pH 7.4) containing 150 mM NaCl and 0.01% Tween 80 were utilized.

Because of the high potency of TPO in the Ba/F3 assay (half maximal stimulation is achieved at approximately 3 pg/ml), it is possible to obtain biologically active material utilizing many different buffer, detergent and redox conditions. However, under most conditions only a small amount of properly folded material (<10%) is obtained. For commercial manufacturing processes, it is desirable to have refolding yields at least 10%, more preferably 30-50% and most preferably >50%. Many different detergents (Triton X-100, dodecyl-beta-maltoside, CHAPS, CHAPSO, SDS, sarkosyl, Tween 20 and Tween 80, Zwittergent 3-14 and others) were assessed for efficiency to support high refolding yields. Of these detergents, only the CHAPS family (CHAPS and CHAPSO) were found to be generally useful in the refolding reaction to limit protein aggregation and improper disulfide formation. Levels of

CHAPS greater than 1% were most useful. Sodium chloride was required for best yields, with the optimal levels between 0.1 M and 0.5M. The presence of EDTA (1-5 mM) limited the amount of metal-catalyzed oxidation (and aggregation) which was observed with some preparations. Glycerol concentrations of greater than 15% produced the optimal refolding conditions. For maximum yields, it was essential to have both oxidized and reduced glutathione or oxidized and reduced cysteine as the redox reagent pair. Generally higher yields were observed when the mole ratio of oxidized reagent is equal to or in excess over the reduced reagent member of the redox pair. pH values between 7.5 and about 9 were optimal for refolding of these TPO variants. Organic solvents (*e.g.* ethanol, acetonitrile, methanol) were tolerated at concentrations of 10-15% or lower. Higher levels of organic solvents increased the amount of improperly folded forms. Tris and phosphate buffers were generally useful. Incubation at 4°C also produced higher levels of properly folded TPO.

Refolding yields of 40-60% (based on the amount of reduced and denatured TPO used in the refolding reaction) are typical for preparations of TPO that have been purified through the first C4 step. Active material can be obtained when less pure preparations (*e.g.* directly after the Superdex 200 column or after the initial refractile body extraction) although the yields are less due to extensive precipitation and interference of non-TPO proteins during the TPO refolding process.

Since TPO (Met^{-1} 1-153) contains 4 cysteine residues, it is possible to generate three different disulfide versions of this protein:

version 1: disulfides between cysteine residues 1-4 and 2-3

version 2: disulfides between cysteine residues 1-2 and 3-4

version 3: disulfides between cysteine residues 1-3 and 2-4

During the initial exploration in determining refolding conditions, several different peaks containing the TPO protein were separated by C4 reversed phase chromatography. Only one of these peaks had significant biological activity as determined using the Ba/F3 assay. Subsequently, the refolding conditions were optimized to yield preferentially that version. Under these conditions, the misfolded versions are less than 10-20% of the total monomer TPO obtained.

The disulfide pattern for the biologically active TPO has been determined to be 1-4 and 2-3 by mass spectrometry and protein sequencing(*i.e.* version 1). Aliquots of the various C4-resolved peaks (5-10 nmoles) were digested with trypsin (1:25 mole ratio of trypsin to protein). The digestion mixture was analyzed by matrix-assisted laser desorption mass spectrometry before and after reduction with DTT. After reduction, masses corresponding to most of the larger tryptic peptides of TPO were detected. In the un-reduced samples, some of these masses were missing and new masses were observed. The mass of the new peaks corresponded basically to the sum of

the individual tryptic peptides involved in the disulfide pair. Thus it was possible to unequivocally assign the disulfide pattern of the refolded, recombinant. biologically active TPO to be 1-4 and 2-3. This is consistent with the known disulfide pattern of the related molecule erythropoietin

D. *Biological activity of recombinant, refolded TPO (met 1-153)*

Refolded and purified TPO (Met^{-1} 1-153) has activity in both *in vitro* and *in vivo* assays. In the Ba/F3 assay, half-maximal stimulation of thymidine incorporation into the Ba/F3 cells was achieved at 3.3 pg /ml (0.3 pM). In the *mpl* receptor-based ELISA, half-maximal activity occurred at 1.9 ng/ml (120 pM). In normal and myelosuppressed animals produced by near-lethal X-radiation, TPO (Met^{-1} 1-153) was highly potent (activity was seen at doses as low as 30 ng/mouse) to stimulate the production of new platelets

EXAMPLE 23

Production of Other Biologically Active TPO Variants in E. coli

Three different TPO variants produced in E. coli, purified and refolded into biological active forms are provided below

(1) MLF - 13 residues from the bacterial-derived signal sequence STII are fused to the N-terminal domain of TPO (residues 1-155). The resulting sequence is

MKKNIAFLLNAYASPAPPAC · ·CVRRA (SEQ ID NO. 85)

where the leader sequence is underlined and C · ·C represents Cys^{7} through Cys^{151}. This variant was constructed to provide a tyrosine for radio-iodination of TPO for receptor and biological studies.

(2) H8MLF - 7 residues from the STII sequence, 8 histidine residues and the Factor Xa enzymatic cleavage sequence IEGR are fused to the N-terminal domain (residues 1-155) of TPO. The sequence is

MKKNIAFHHHHHHHHIEGRSPAPPAC·····CVRRA (SEQ ID NO: 86)

where the leader sequence is underlined and C·····C represents Cys^{7} through Cys^{151}. This variant, when purified and refolded, can be treated with the enzyme Factor Xa which will cleave after the arginine residue of the sequence IEGR yielding a TPO variant of 155 residues in length with a natural serine N-terminal amino acid.

(3) T-H8MLF - is prepared as described above for variant (2), except a thrombin sensitive sequence IEPR is fused to the N-terminal domain of TPO. The resulting sequence is

MKKNIAFHHHHHHHHIEPRSPAPPAC·····CVRRA (SEQ ID NO: 87)

where the leader sequence is underlined and and C·····C represents Cys^{7} through Cys^{151}. This variant, after purification and refolding can be treated with the enzyme thrombin to generate a natural N-terminal variant of TPO of 155 residues in length.

A. Recovery, solubilization and purification of monomeric, biologically active TPO variants (1), (2), and (3).

All of the variants were expressed in E. coli. The majority of the variants were found in refractile bodies, as observed in **Example 22** for TPO (Met^{-1} 1-153). Identical procedures for the recovery, solubilization and purification of monomeric TPO variants was achieved as described in **Example 22.** Identical refolding conditions to those used for TPO (Met^{-1} 1-153) were used with overall yields of 30-50%. After refolding, the TPO variants were purified by C4 reversed phase chromatography in 0.1% TFA utilizing an acetonitrile gradient as described previously. All of the TPO variants (in their unproteolyzed forms) had biological activity as assessed by the Ba/F3 assay with half-maximal activities of 2-5 pM.

B. Proteolytic processing of Variants (2) and (3) to generate authentic N-terminal TPO (1-155).

TPO variants **(2)** and **(3)** above were designed with an enzymatically-cleavable leader peptide before the normal N-terminal amino acid residue of TPO. After refolding and purification of variants **(2)** and **(3)** as described above, each was subjected to digestion with the appropriate enzyme For each variant, the acetonitrile from the C4 reversed phase step was removed by blowing a gentle stream of nitrogen on the solution. Thereafter the two variants were treated with either Factor Xa or thrombin as described below

For TPO variant **(2)**, 1 M Tris buffer, pH 8, was added to the acetonitrile-free solution to a final concentration of 50 mM and the pH was adjusted to 8 if necessary. NaCl and $CaCl_2$ were added to 0.1 M and 2 mM, respectively. Factor Xa (New England Biolabs) was added to achieve about a 1:25 to 1:100 mole ratio of enzyme to variant. The sample was incubated at room temperature for 1-2 hr. to achieve maximal cleavage as assessed by a change in migration on SDS gels representing the loss of the leader sequence. Thereafter, the reaction mixture was purified by C4 reversed phase chromatography using the same gradient and conditions as described above for the purification of properly folded variants. Uncleaved variant B was separated from cleaved variant **(2)** by these conditions. The N-terminal amino acids were shown to be SPAPP, indicating that removal of the N-terminal leader sequence was successful. Factor Xa also generated variable amounts of an internal cleavage within the TPO domain; cleavage was observed after the arginine residue at position number 118 generating an additional N-terminal sequence of TTAHKDP (SEQ ID NO: 88). On non-reducing SDS gels, a single band at approximately 17000 daltons was observed for the Factor Xa cleaved variant: on reducing gels two bands were seen of molecular weight of approximately 12000 and 5000 daltons, consistent with cleavage at arginine 118. This observation also confirmed that the two parts of the molecule were held together

by a disulfide bond between the 1st and 4th cysteine residues, as deduced from the tryptic digestion experiments described above. In the Ba/F3 biological assay, the purified TPO (1-155) variant, after removal of the N-terminal leader sequence and with the internal cleavage, had a half-maximal activity of 0.2 to 0.3 picomolar. The intact variant with the leader sequence had a half-maximal activity of 2-4 picomolar.

For variant (3), the digestion buffer consisted of 50 mM Tris, pH 8, 2% CHAPS, 0.3 M NaCl, 5 mM EDTA and human or bovine thrombin (Calbiochem) at a 1:25 to 1:50 by weight of enzyme to TPO variant protein. Digestion was conducted at room temperature for 2-6 hours. The progress of the digestion was assessed by SDS gels as described above for the Factor Xa cleavage reaction. Generally, more than 90% cleavage of the leader sequence was achieved in this time. The resultant TPO was purified on C4 reversed phase columns as described above and was shown to have the desired N-terminal by amino acid sequencing. Only very minor (<5%) amounts of an internal cleavage at the same arginine-threonine bond as observed above with Factor Xa was obtained. The resultant TPO protein had high biological activity with half-maximal responses in the Ba/F3 assay at 0.2-0.4 picomolar protein. In the *mpl* receptor based ELISA, this protein had a half-maximal response at 2-4 ng/ml purified protein (120-240 picomolar) while the intact variant containing the leader sequence was less potent in both assays by 5-10 fold. For animal studies, the HPLC-purified cleaved protein was dialyzed into physiological acceptable buffers, with 150 mM NaCl, 0.01% Tween 80 and 10 mM sodium succinate pH 5.5, or 10 mM sodium acetate, pH 5.5, or 10 mM sodium phosphate pH 7.4. By HPLC and SDS gels, the purified protein was stable for several weeks when stored at 4°C. In normal and myelosuppressed mice, this purified TPO with the authentic N-terminal sequence was highly active, stimulating the production of platelets at doses as low as 30 ng/mouse.

EXAMPLE 24

Synthetic mpl Ligand

Although Human *mpl* ligand (hML) is usually made using recombinant methods, it can also be synthesized via enzymatic ligation of synthetic peptide fragments using methods described below. Synthetic production of hML allows the incorporation of unnatural amino acids or synthetic functionalities such as polyethylene glycol. Previously a mutant of the serine protease subtilisin BPN, subtiligase (S221C/P225A) was engineered to efficiently ligate peptide esters in aqueous solution (Abrahmsen *et al., Biochem.*, 30:4151-4159 [1991]). It has now been shown that synthetic peptides can be enzymatically ligated in a sequential manor to produce enzymatically active long peptides and proteins such as ribonuclease A (Jackson *et al., Science,* [1994]). This technology, described in more detail below, has enabled us to

chemically synthesize long proteins that previously could be made only with recombinant DNA technology.

A general strategy for hML_{153} synthesis using subtiligase is shown **(Scheme 1)**. Beginning with a fully deprotected peptide corresponding to the C-terminal fragment of the protein, an N-terminal protected, C-terminal activated ester peptide is added along with subtiligase. When the reaction is complete, the product is isolated by reverse phase HPLC and the protecting group is removed from the N-terminus. The next peptide fragment is ligated, deprotected and the process is repeated using successive peptides until full length protein is obtained. The process is similar to solid phase methodology in that an N-terminal protected C-terminal activated petide is ligated to the N-terminus of the preceding peptide and protein is synthesized in a C->N direction. However because each coupling results in addition of up to 50 residues and the products are isolated after each ligation, much longer highly pure proteins can be synthesized in reasonable yields.

Scheme 1. Strategy for Synthesis of hML Using Subtiligase

$$\text{R-NH-}\mathbf{Peptide_2}\text{-CO-R'} + \text{H}_2\text{N-}\mathbf{Peptide_1}\text{-CO}_2$$

$$\downarrow \text{1) Subtiligase}$$

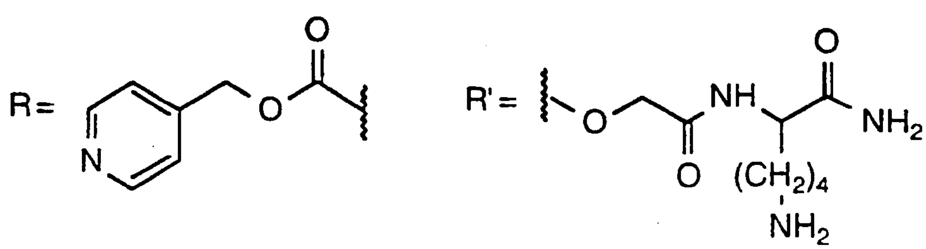
$$\text{R-NH-}\mathbf{Peptide_2}\text{-CO-NH-}\mathbf{Peptide_1}\text{-CO}_2$$

$$\downarrow \text{2) Zn/CH}_3\text{CO}_2\text{H}$$

$$\text{H}_2\text{N-}\mathbf{Peptide_2}\text{-CO-NH-}\mathbf{Peptide_1}\text{-CO}_2$$

$$\downarrow \text{3) repeat 1 + 2}$$

$$\text{H}_2\text{N-}\mathbf{Peptide_3}\text{-CO-NH-}\mathbf{Peptide_2}\text{-CO-NH-}\mathbf{Peptide_1}\text{-CO}_2$$



Based on our knowledge of the sequence specificity of the subtiligase as well as the amino acid sequence of the biologically active "epo-domain" of hML, we divided hML_{153} into seven fragments 18-25 residues in length. Test ligation tetrapeptides were synthesized to determine suitable ligation junctions for the 18-25mer's. **Table 13** shows the results of these test ligations.

TABLE 13

hML Test Ligations. Donor and nucleophile peptides were dissolved at 10 mM in 100 mM tricine (pH 7.8) at 22°C. Ligase was added to a final concentration of 10 µM from a 1.6 mg/mL stock (~70 µM) and the ligation allowed to proceed overnight. Yields are based on % ligation vs. hydrolysis of the donor peptides.

Site	Donor (glc-K-NH_2)	Nucleophile-NH_2	%Hydrolysis	%Ligation
1 (23/24)	HVLH (SEQ ID NO: 89)	SRLS (SEQ ID NO: 90)	92	08
(22/23)	SHVL (SEQ ID NO: 91)	HSRL (SEQ ID NO: 92)	48	52
2 (46/47)	AVDF (SEQ ID NO: 93)	SLGE (SEQ ID NO: 94)	22	78
3 (69/70)	AVTL (SEQ ID NO: 95)	LLEG (SEQ ID NO: 96)	53	47
4 (89/90)	LSSL (SEQ ID NO: 97)	LGQL (SEQ ID NO: 98)	95	05
(88/89)	C(acm)LSS (SEQ ID NO: 99)	LLGQ (SEQ ID NO: 100)	00	00
(90/91)	SSLL (SEQ ID NO: 101)	GQLS (SEQ ID NO: 102)	45	55
(88/89)	CLSS (SEQ ID NO: 103)	LLGQ (SEQ ID NO: 100)	90	10
5(107/108)	LQSL (SEQ ID NO: 104)	LGTQ (SEQ ID NO: 105)	99	01
(106/107)	ALQS (SEQ ID NO: 106)	LLGT (SEQ ID NO: 107)	70	30
6(128/129)	NAIF (SEQ ID NO: 108)	LSFQ (SEQ ID NO: 109)	60	40

Based on these experiments, the ligation peptides indicated in **Table 14** should be efficiently ligated by the subtiligase. A suitable protecting group for the N-terminus of each donor ester peptide was needed to prevent self-ligation. We chose an isonicotinyl (iNOC) protecting group (Veber *et al., J. Org. Chem.*, 42:3286-3289 [1977]) because it is water soluble, it can be incorporated at the last step of solid phase peptide synthesis and it is stable to anhydrous HF used to deprotect and cleave peptides from the solid phase resin. In addition, it can be removed from the peptide after each ligation under mild reducing conditions (Zn/CH_3CO_2H) to afford a free N-terminus for subsequent ligations. A glycolate-lysyl-amide (glc-K-NH_2) ester was used for C-terminal activation based on previous experiments which showed this to be efficiently acylated by subtiligase (Abrahmsen *et al., Biochem.*, 30:4151-4159 [1991]). The iNOC-protected, glc-K-amide activated peptides can be synthesized using standard solid phase methods as outlined **(Scheme 2)**. The peptides are then seqentially ligated until the full protein is produced and the final product refolded *in vitro*. Based on homology with EPO, disulfide pairs are believed to be formed between cysteine residues 7 and 151 and between 28 and 85. Oxidation of the disulfides may be accomplished by simply stirring the reduced material under an oxygen atmosphere for several hours. The refolded material can then be purified by HPLC and fractions containing active protein pooled and lyophilized. As an alternative, disulfides can be differentially protected to control sequential oxidation between specific disulfide pairs. Protection of cysteines 7 and 151 with acetamidomethyl (acm) groups would ensure oxidation of 28 and 85. The acm groups could then be removed and residues 7 and 151 oxidized. Conversely, residues 28 and 85 could be acm protected and oxidized in case sequential oxidation is required for correct folding. Optoinally, Cysteins 28 and 85 may be substituted with another natural or unnatural residue other than Cys to insure proper oxidation of cysteins 7 and 151.

TABLE 14.

Peptide Fragments Used For Total Synthesis of h-ML Using Subtiligase

__Fragment__

__Sequence__

1 (SEQ ID NO: 110)
iNOC-HN-SPAPPACDLRVLSKLLRDSHVL-glc-K-NH_2 (1-22)

2 (SEQ ID NO: 111)
iNOC-HN-HSRLSQCPEVHPLPTPVLLPAVDF-glc-K-NH_2 (23-46)

3 (SEQ ID NO: 112)

iNOC-HN-SLGEWKTQMEETKAQDILGAVTL-glc-K-NH_2 (47-69)

4 (SEQ ID NO: 113)

iNOC-HN-LLEGVMAARGQLGPTCLSSLL-glc-K-NH_2 (70-90)

5 (SEQ ID NO: 114)

iNOC-HN-GQLSGQVRLLLGALQS-glc-K-NH_2 (90-106)

6 (SEQ ID NO: 115)

iNOC-HN-LLGTQLPPQGRTTAHKDPNAIF-glc-K-NH2 (107-128)

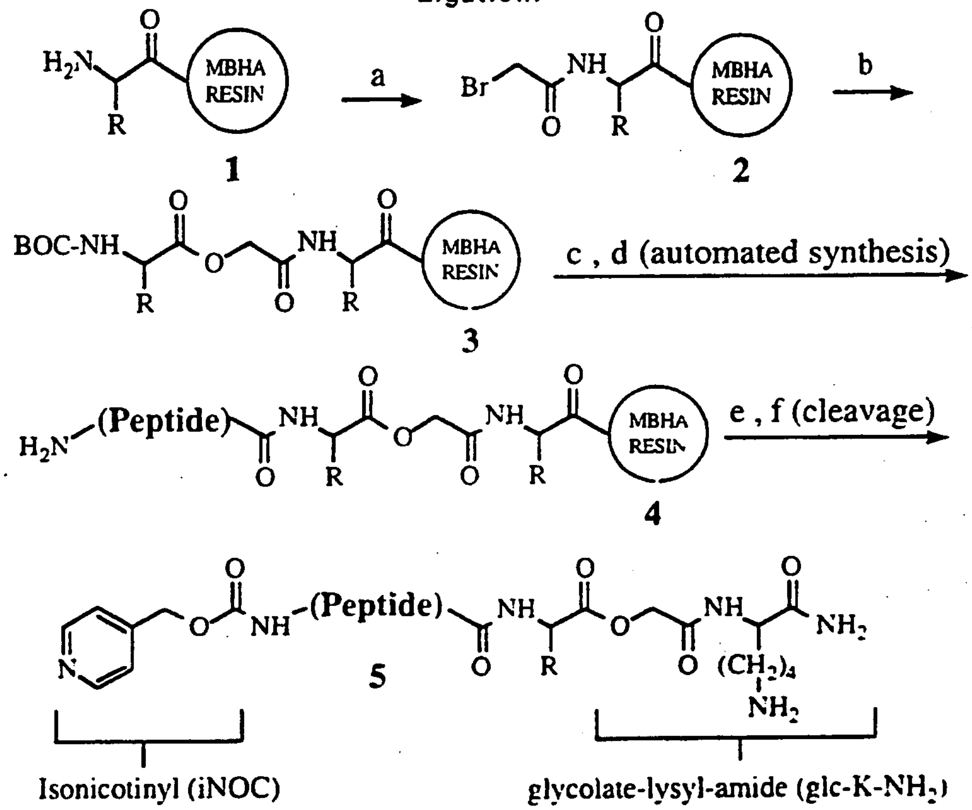
7 (SEQ ID NO: 116)

H_2N-LSFQHLLRGKVRFLMLVGGSTLCVR-CO_2 (129-153)

Peptide ligations are carried out at 25°C in 100mM tricine, pH 8 (freshly prepared and degassed by vacuum filtration through a 5 µM filter). Typically the C-terminal fragment is dissolved in buffer (2-5 mM peptide) and a 10x stock solution of subtiligase (1 mg/ml in 100mM tricine, pH 8) is added to bring the final enzyme concentration to ~ 5µM. A 3-5 molar excess of the glc-K-NH_2 activated donor peptide is then added as a solid dissolved and the mixture allowed to stand at 25°C. The ligations are monitored by analytical reverse phase C18 HPLC (CH_3CN/H_2O gradient with 0.1% TFA). The ligation products are purified by preparative HPLC and lyophilized. Isonicotinyl (iNOC) deprotection was performed by stirring HCl activated zinc dust with the protected peptide in acetic acid. The zinc dust is removed by filtration and the acetic acid evaporated under vacuum. The resulting peptide can be used directly in the next ligation and the process is repeated. Synthetic hML_{153} can be ligated by procedures analogous to those described above to synthetic or recombinant $hML_{154-332}$ to produce synthetic or semisynthetic full length hML.

Synthetic hML has many advantages over recombinant. Unnatural side chains can be introduced in order to improve potency or specificity. Polymer functionalities such as polyethylene glycol can be incorporated to improve duration of action. For example, polyethylene glycol can be attached to lysine residues of the individual fragments (**Table 14**) before or after one or more ligation steps have been performed. Protease sensitive peptide bonds can be removed or altered to improve stability *in vivo*. In addition, heavy atom derivatives can be synthesized to aid in structure determination.

Scheme 2. Solid Phase Synthesis of Peptide Fragments for Segment Ligation.



a) Lysyl-paramethylbenzhydrylamine (MBHA) resin **1** (0.63 meq./gm., Advanced ChemTech) is stirred with bromoacetic acid (5 eq.) and diisopropyl carbodiimide (5 eq.) for 1 h. at 25°C in dimethylacetamide (DMA) to afford the bromoacetyl derivative **2**. b) The resin is washed extensively with DMA and individual Boc-protected amino acids (3 eq., Bachem) are esterified by stirring with sodium bicarbonate (6 eq.) in dimethylformamide (DMF) for 24 h. at 50°C to afford the corresponding glycolate-phenylalanyl-amide-resin **3**. The amino acetylated resin **3** is washed with DMF (3x) and dichloromethane (CH_2Cl_2) (3x) and can be stored at room temperature for several months. The resin 3 can then be loaded into an automated peptide synthesizer (Applied Biosystems 430A) and the peptides elongated using standard solid phase procedures (5). c) The N-α-Boc group is removed with a solution of 45% trifluoroacetic acid in CH_2Cl_2. d) Subsequent Boc-protected amino acids (5 eq.) are preactivated using benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP, 4 eq.) and N-methylmorpholine (NMM, 10 eq.) in DMA and coupled for 1-2 h. e) The final N-α-Boc group is removed (TFA/CH_2Cl_2) to afford **4** and the isonicotinyl (iNOC) protecting group is introduced as described previously (4) via stirring with of

4-isonicotinyl-2-4-dinitrophenyl carbonate (3 eq.) and NMM (6 eq.) in DMA at 25°C for 24 h. f) Cleavage and deprotection of the peptide via treatment with anhydrous HF (5% anisole/ 5% ethylmethyl sufide) at 0°C for 1 h. affords the iNOC-protected, glycolate-lys-amide activated peptide 5 which is purified by reverse phase C18 HPLC (CH_3CN/H_2O gradient. 0.1% TFA). The identity of all substrates is confirmed by mass spectrometry.

* * * * *

SUPPLEMENTAL ENABLEMENT

The invention as claimed is enabled in accordance with the above specification and readily available references and starting materials. Nevertheless, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the cell line listed below:

Escherichia coli, DH10B-pBSK - *hmpl* 1.8, ATCC accession no. CRL 69575, deposited February 24, 1994.

Plasmid, pSVI5.ID.LL.MLORF ATCC accession no. CRL _____, deposited December ____, 1994; and

CHO DP-12 cells, ML 1/50 MCB (labeled #1594), ATCC accession no. CRL 11770, deposted December 6, 1994

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treat, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

* * * * *

While the invention has necessarily been described in conjunction with preferred embodiments and specific working examples, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein, without departing from the spirit and scope thereof. Hence, the invention can be practiced in ways other than those specifically described herein. It is therefore intended that the protection granted by letters patent hereon be limited only by the appended claims and equivalents thereof.

All references cited herein are hereby expressly incorporated by reference.

CLAIMS

We claim:

1. An isolated substantially homogeneous *mpl* ligand polypeptide.

2. The *mpl* ligand polypeptide of Claim 1 selected from the group consisting of
   (a) a fragment polypeptide;
   (b) a variant polypeptide; and
   (c) a chimeric polypeptide.

3. The *mpl* ligand polypeptide of Claim 1 selected from the group consisting of
   (a) the polypeptide that is isolated from a mammal;
   (b) the polypeptide that is made by recombinant means; and
   (c) the polypeptide that is made by synthetic means.

4. The *mpl* ligand polypeptide of Claim 1 selected from the group consisting of
   (a) the polypeptide that is human; and
   (b) the polypeptide that is non-immunogenic in a human.

5. An isolated substantially homogeneous *mpl* agonist characterized in that:

(a) the agonist stimulates the incorporation of labeled nucleotides (^{3}H-thymidine) into the DNA of IL-3 dependent Ba/F3 cells transfected with human *mpl* P ; or

(b) the agonist stimulates ^{35}S incorporation into circulating platelets in a platelet rebound assay.

6. A fragment polypeptide according to Claim 2 represented by

X-hTPO(7-151)-Y

Where

hTPO(7-151) represents the human TPO (hML) amino acid sequence from Cys^7 through Cys^{151} inclusive;

X represents an amino group of Cys^7 or amino-terminus amino acid residue(s) selected from the group

M,
MA,
MPA,
MPPA, (SEQ ID NO: 117)
MAPPA, (SEQ ID NO: 118)

MPAPPA. (SEQ ID NO: 119)
MSPAPPA, (SEQ ID NO: 120)
A,
PA,
PPA,
APPA. (SEQ ID NO: 121)
PAPPA, (SEQ ID NO: 122)
SPAPPA, (SEQ ID NO: 123)

Y represents the carboxy terminal group of Cys^{151} or carboxy-terminus amino acid residue(s) selected from the group

V,
VR.
VRR.
VRRA. (SEQ ID NO: 124)
VRRAP. (SEQ ID NO: 125)
VRRAPP. (SEQ ID NO: 126)
VRRAPPT. (SEQ ID NO: 127)
VRRAPPTT. (SEQ ID NO: 128)
VRRAPPTTA. (SEQ ID NO: 129)
VRRAPPTTAV (SEQ ID NO: 130)
VRRAPPTTAVP. (SEQ ID NO: 131)
VRRAPPTTAVPS. (SEQ ID NO: 132)
VRRAPPTTAVPSR, (SEQ ID NO: 133)
VRRAPPTTAVPSRT. (SEQ ID NO: 134)
VRRAPPTTAVPSRTS. (SEQ ID NO: 135)
VRRAPPTTAVPSRTSL. (SEQ ID NO: 136)
VRRAPPTTAVPSRTSLV. (SEQ ID NO: 137)
VRRAPPTTAVPSRTSLVL. (SEQ ID NO: 138)
VRRAPPTTAVPSRTSLVLT. (SEQ ID NO: 139)
VRRAPPTTAVPSRTSLVLTL. (SEQ ID NO: 140)
VRRAPPTTAVPSRTSLVLTLN. (SEQ ID NO: 141)
VRRAPPTTAVPSRTSLVLTLNE. (SEQ ID NO: 142)
VRRAPPTTAVPSRTSLVLTLNEL. (SEQ ID NO: 143)
VRRAPPTTAVPSRTSLVLTLNELP. (SEQ ID NO: 144)

and amino-terminus amino acid residue(s) extensions comprising one or more of the residues 176-332 of human ML as provided in **Fig. 1** (SEQ ID NO: 1).

7. A fragment polypeptide according to Claim 6 selected from the group TPO(1-153) and
TPO(1-245).

8. A fragment polypeptide according to Claim 2, wherein the amino acid sequence of the fragment polypeptide comprises
SPAPPACDLRVLSKLLRDSHVL,
(SEQ ID NO: 110)
HSRLSQCPEVHPLPTPVLLPAVDF,
(SEQ ID NO: 111)
SLGEWKTQMEETKAQDILGAVTL,
(SEQ ID NO: 112)
LLEGVMAARGQLGPTCLSSLL,
(SEQ ID NO: 113)
GQLSGQVRLLLGALQS,
(SEQ ID NO: 114)
LLGTQLPPQGRTTAHKDPNAIF,
(SEQ ID NO: 115)
LSFQHLLRGKVRFLMLVGGSTLCVR, and
(SEQ ID NO: 116)
combinations thereof.

9. The polypeptide of Claim 6 that is unglycosylated.

10. An isolated polypeptide encoded by a nucleic acid having a sequence that hybridizes under moderately stringent conditions to the nucleic acid molecules having a nucleic acid sequence provided in **Fig. 1** (SEQ ID NO: 2).

11. The polypeptide of Claim 11 that is biologically active.

12. The polypeptide of Claim 1 selected from the group hML, hML_{153}, hML(R153A, R154A), hML2, hML3, hML4, mML, mML2, mML3, pML, and pML2.

13. A polypeptide according to Claim 2, wherein the amino acid sequence of the polypeptide comprises amino acid residues 1 to X of **Fig. 1** (SEQ ID NO: 1), where X is selected from the group 153, 155, 164, 174, 191, 205, 207, 217, 229, 245 and 332.

14. An isolated substantially homogeneous *mpl* ligand polypeptide sharing at least 80% sequence identity with the polypeptide of Claim 13.

15. The polypeptide of Claim 13 wherein X is 153.

16. A chimera comprising the *mpl* ligand of Claim 13 fused to a heterologous polypeptide.

17. The chimera of Claim 16 wherein the heterologous polypeptide is an immunoglobin polypeptide

18. The chimera of Claim 16 wherein the heterologous polypeptide is an interlukin polypeptide.

19 A chimera comprising the N-terminus residues 1 to about 153 to 157 of hML substituted with one or more, but not all, of the human EPO residues added or substituted into the N-terminus residues of hML at positions corresponding to the alignment shown in **Fig. 10**.

20. An antibody that is capable of binding the *mpl* ligand polypeptide of Claim 13

21. A hybridoma cell line producing the antibody of Claim 20.

22 An isolated nucleic acid molecule encoding the *mpl* ligand polypeptide of Claim 1.

23. An isolated nucleic acid molecule encoding the *mpl* ligand polypeptide of Claim 13.

24. An isolated nucleic acid molecule comprising the open reading frame nucleic acid sequence shown in **Fig. 1** (SEQ ID NO: 2) .

25. The isolated nucleic acid molecule of Claim 24 encoding a *mpl* ligand polypeptide selected from the group hML, hML_{153}, hML(R153A, R154A), hML2, hML3, hML4, mML, mML2, mML3, pML, and pML2.

26. An isolated nucleic acid molecule selected from the group consisting of

(a) a cDNA clone comprising the nucleotide sequence of the coding region of the *mpl* ligand gene;

(b) a DNA sequence capable of hybridizing under stringent conditions to a clone of (a); and

(c) a genetic variant of any of the DNA sequences of (a) and (b) which encodes a polypeptide possessing a biological property of a naturally occurring *mpl* ligand polypeptide.

27. An isolated DNA molecule having a sequence capable of hybridizing to a DNA sequence provided in **Fig. 1** (SEQ ID NO: 2) under moderately stringent conditions, wherein the DNA molecule encodes a biologically active *mpl* ligand polypeptide.

28. The nucleic acid molecule of Claim 25 further comprising a promoter operably linked to the nucleic acid molecule.

29. An expression vector comprising the nucleic acid sequence of Claim 25 operably linked to control sequences recognized by a host cell transformed with the vector.

30. A host cell transformed with the vector of Claim 29.

31. A process of using a nucleic acid molecule encoding the *mpl* ligand polypeptide to effect production of the *mpl* ligand polypeptide comprising culturing the host cell of Claim 30.

32. The process of Claim 31 wherein the *mpl* ligand polypeptide is recovered from the host cell.

33. The process of Claim 31 wherein the *mpl* ligand polypeptide is recovered from the host cell culture medium.

34. A method of determining the presence of *mpl* ligand polypeptide, comprising hybridizing DNA encoding the *mpl* ligand polypeptide to a test sample nucleic acid and determining the presence of *mpl* ligand polypeptide DNA.

35. A method of amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase reaction with nucleic acid encoding a *mpl* ligand polypeptide.

36. A composition comprising the *mpl* ligand polypeptide of Claim 1 and a pharmaceutically acceptable carrier.

37. A method for treating a mammal having or at risk for thrombocytopenia comprising administering to a mammal in need of such treatment a therapeutically effective amount of the composition of Claim 36.

38. The composition of Claim 36 further comprising a therapeutically effective amount of an agent selected from the group consisting of a cytokine, colony stimulating factor, and interleukin.

39. The composition of Claim 38 wherein the agent is selected from KL, LIF, G-CSF, GM-CSF, M-CSF, EPO IL-1, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-9 and IL-11.

40. A polypeptide or fragment of a polypeptide substantially as described herein with reference to the accompanying figures.

41. An isolated nucleic acid molecule substantially as described herein with reference to the accompanying figures.

42. A method of determining the presence of *mpl* ligand polypeptide substantially as described herein with reference to the accompanying figures.

43. A pharmaceutical composition substantially as described herein with reference to the accompanying figures.

Patents Act 1977 **Examiner's report to the Comptroller under Section 17** **(The Search report)**	Application number GB 9425831.6
Relevant Technical Fields (i) UK Cl (Ed.N) C3H (HAS, HB7P, HFZ) (ii) Int Cl (Ed.6) C07K 14/475; C12N 15/12	Search Examiner MR C SHERRINGTON Date of completion of Search 20 MARCH 1995
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASES: WPI, CLAIMS, DIALOG/BIOTECH	Documents considered relevant following a search in respect of Claims :- 1 to 36, 38 to 43

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Category	Identity of document and relevant passages	Relevant to claim(s)
X	WO 90/12877 A1 (CETUS CORPORATION) whole document, especially page 4, lines 26 to 33; Claims 3, 57, 81	1 to 5,22, 26,29 to 33, 38,39 (at least)
X	WO 93/11247 A1 (GENENTECH INC) whole document, especially page 14, line 27; Claims 12, 13, 19	1 to 5,22, 26,29 to 33 (at least)
X	US 5223408 (GENENTECH INC) whole document, especially column 6, lines 22 to 23; Claim 9	1 to 5,22, 26,29 to 33 (at least)
X	Int Cong Throm Haem 1979, 42(1), 283, Abs P5-028/0668 Thrombopoietin-induced stimulation of megakaryocyte-enriched bone marrow cultures	1 to 5,22, 26,29 to 33 (at least)
X	Exp Hematol 1989, 17(8), 865-871 A Four-Step Procedure for the Purification of Thrombopoietin	1 to 5,22, 26,29 to 33 (at least)
X	Exp Hematol 1989, 17(8), 903-907 The Effect of Partially Purified Thrombopoietin on Guinea Pig Megakarocyte Ploidy in vitro	1 to 5,22, 26,29 to33 (at least)

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Category	Identity of document and relevant passages	Relevant to claim(s)
X	Am J Pediatr Hematol Oncol 1992, 14(1), 8-21 Thrombopoietin: Ito Biology, Clinical Aspects, and Possibilities	1 to 5,22, 26,29 to 33, 36 (at least)
P,X	Stem Cells 1994, 12(6), 586-598 The Structure, Biology and Potential Therapeutic Applications of Recombinant Thrombopoietin	1 to 5,22, 26,29 to 33, 36,38,39
P,X	Proc Natl Acad Sci USA 1994, 91(26), 13023-13027 Human thrombopoietin: Gene structure, cDNA sequence, expression and chromosomal location	1 to 5,22, 26,29 to 33
P,X	Nature 1994, 369 (6481), 565-568 Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo	1 to 5,22, 26,29 to 33
P,X	FEBS Letters 1994, 353(1), 57-61 Molecular cloning and chromosomal location of the human thrombopoietin gene	1 to 5,22, 26,29 to 33

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